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I. SITE OF DECOMPOSITION OF METHYL-BROMIDE IN COCOA BEANS. II. FIELD DESORPTION MASS SPECTROMETRY OF AMINO ACIDS AND PEPTIDES.

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University of Windsor

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I SITE OF DECOMPOSITION OF METHYL BROMIDE IN COCOA BEANS

II FIELD DESORPTION MASS SPECTROMETRY OF AMINO ACIDS AND PEPTIDES

By

S. Asante-Poku

A Dissertation

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September 2, 1975

Professor D. E. Schmidt, Jr.
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Dear Professor Schmidt:

I have read Asante-Poku's thesis carefully and am impressed with the amount of work that your candidate has done and the variety of experience that he has had in the course of his research with you. I have no reservations in approving this thesis as an external examinar.

I had planned to come to Windsor and participate in the oral examination, because, in addition to whatever contribution I might be able to make, I am genuinely interested in the work and would profit by the experience. Unfortunately several unforeseen events have made it impossible for me to leave the Long Island area at this time and the only communication that I can have with you for the present and Mr. Asante-Poku is by mail or phone. I would like to say at this point that if you or your students have the occasion to be in the New York City area and can spare a day to come approximately 70 miles east of Manhattan Island I would be most happy to have you visit Brookhaven and perhaps discuss science of mutual interest.

With respect to Mr. Asante-Poku's thesis and oral examination I assume that the philosophy of the examination is to insure that the candidate having mastered certain techniques and scientific disciplines does not leave with the feeling that his education is complete. In this spirit he might be asked, How would he go about measuring the actual temperature of the molecules adsorbed on the tips of the emitters in a field desorption ion source? This is a problem of deep concern to Professor Beckey's group and they have invested some time in looking into the possibility of measuring melting points of known material dispersed on the emitter needles. The rub of course is that with particles small enough to fit on the end of a needle the surface to volume ratio is large and one deals with the physical properties of a colloid rather than a macroscopic crystal. Mr. Asante-Poku's background in organic chemistry may serve him well and he may be aware, from experience of the effect of particle size on melting point. On the other hand a sound knowledge of physical chemistry would lead to an awareness of the effect of surface energy on the energy required to produce the disorder of the molten material. This is a topic that could be

developed for discussion that bears on the technique of field desorption and its practical exploitation.

A second area that might test the candidate's understanding of the working of a mass spectrometer deals with the collection efficiency of field desorbed ions. Working in collaboration with Dr. Winkler we have studied the effect of floating a field desorption source at a higher voltage then the normal +3 -7 combination. This technique is facilitated by use of an electromagnetic separater magnet that will resolve ions with KE up to 60 kilovolts. Significantly higher sensitivity can be achieved with ions extracted from the field desorption source with energies of 20 keV over the much lower voltages that produce the ions directly from the emitter. A discussion of this problem would test Mr. Asante-Poku's grasp of the details of the ion optics involved in the field emission process which should at least have him recognize the losses which are encountered in focusing ions pulled off a needle in a variety of directions not necessarily coincident with the axis of the focused beam.

I realize that I have emphasized topics that are primarily physical rather than organic or biochemical in nature and this may be emphasizing the candidate's minor rather than major interest. I feel that at this stage in his development he would be well advised to strengthen areas that may not have been emphasized in his training.

I hope that you find these remarks useful and that some of the conclusions that I have drawn above in a state of benign ignorance are not presumptuous. It is a good thesis, well directed and fine work. I offer my congratulations to the candidate.

Sincerely,

Lewis Friedman

LF:dp

ABSTRACT

PART I

The main site of decomposition of methyl bromide in cocoa beans was shown to be in the alcohol-insoluble proteins of the shells. The methyl group of the fumigant becomes covalently bonded to the α-amino group of the various amino acids, the imidazole ring of histidine, and the ϵ-amino group of lysine. An amino acid analysis of cocoa beans shows that methionine is the limiting essential amino acid and that cysteine is concentrated in the alcohol-insoluble proteins of the nib.

PART II

The field desorption mass spectra of seven amino acids, five dipeptides, three tripeptides, two tetrapeptides, a pentapeptide, a hexapeptide and a nonapeptide, bradykinin, were investigated. In each case a molecular $[M]^+$ and/or a quasimolecular $[M+1]^+$ mass peak was obtained. Sufficient fragmentation of the peptide backbone occurred to allow a sequence determination of all the peptides. The pentapeptide produced $[M]^+$, $[M+1]^+$ and $[M+2]^+$ ions which also included molecules of ethanol, water and/or ethylacetate bond to the ion. The origin of the hydrogens which add to the peptides to produce $[M+1]^+$ and $[M+2]^+$ arise from another peptide molecule.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. D. E. Schmidt Jr. for his guidance, concern and patience during the course of this work.

I would also like to express my appreciation and thanks to Dr. W. P. Aston and Professor G. W. Wood for their help. I wish to thank my fellow graduate students particularly Mr. P. Y. Lau for their assistance and encouragement.

I also wish to acknowledge the financial support of the Canadian Commonwealth Scholarship and Fellowship Committee as well as a teaching Assistantship from the Department of Chemistry, the University of Windsor.

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ABBREVIATIONS

c.i.	chemical ionization
c.i.m.s	chemical ionization mass spectrometry
ca	about
e.i.m.s	electron impact mass spectrometry
f.d.	field desorption
f.d.m.s.	field desorption mass spectrometry
f.i.	field ionization
f.i.m.s.	field ionization mass spectrometry
Gly	Glycine
Ala	Alanine
Val	Valine
Leu	Leucine
Ile	Isoleucine
Ser	Serine
Thr	Threonine
Phe	Phenylalanine
Tyr	Tyrosine
Trp	Tryptophan
Cys	Cysteine
$(Cys)_2$	Cystine
Met	Methionine
Pro	Proline
Asp	Aspartic Acid

Glu	Glutamic Acid
His	Histidine
Arg	Arginine
Lys	Lysine
NCBZ	N-Carbobenzyloxy

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PART I

SITE OF DECOMPOSITION

OF METHYL BROMIDE IN COCOA BEANS

CHAPTER I

INTRODUCTION

Methyl bromide is used as a gaseous pesticide to control infestations of insects, mites, rodents and to a lesser extent bacteria, yeast and molds in stored agricultural commodities. It is also used for quarantine purposes on a wide range of fresh fruits and vegetables and in the treatment of soil against nematodes, soil-borne insects, and fungi.

Under normal fumigation conditions methyl bromide is physically sorbed (1); the amount depends upon the concentration of fumigant during treatment, nature of the substrate, temperature, moisture content of the food, the period of exposure to fumigant, and the amount of ventillation allowed (2).

After fumigation the physically sorbed fumigant is rapidly given off into the atmosphere, some methyl bromide is, however, incorporated chemically into the agricultural product. Inorganic bromide and the organic methyl group irreversibly bonded to some constituents of the commodity are the resulting products. The reactions leave a "fixed" residue of bromide which is in the water-soluble ionic form (3).

A considerable amount of attention has been given to the amount of inorganic bromide formed in

this way and to the setting of tolerances for inorganic bromide in agricultural products which includes milk (4), cereals (5-10, 11-13), cocoa beans (14, 15), vegetables and fruits (16, 17) and cattle (18). Tolerances (19) for total bromide ion in foods have previously been proposed primarily to prevent the excessive use of certain bromine-containing pesticides rather than a means of limiting the intake of bromide ion in the diet. However, where a large addition to the bromide ion content of a food results from the post-harvest use of a bromine-containing fumigant such as methyl bromide, this addition provides an indication of the extent of the reaction of the fumigant with the food and an indirect measure of the amount of methylated and/or other organic reaction products that may be present (20). The excessive formation of these residues may be as important as, and might possibly be more important than, the regulation of the bromide ion content per se (6).

The persistence in agricultural commodities of small amounts of methyl bromide has been reported (21-23). Scudamore and Heuser (23) have presented data for a wide range of dry foods showing the rate of disappearance of methyl bromide under controlled conditions from both sealed samples and well ventilated samples. For most products held at 25° the amount

of residual methyl bromide falls below 1 ppm within a few days. The rate of loss is, however, lower in groundnuts and cocoa beans (23).

Less attention has been given to study of the fate of the organic moiety of methyl bromide and hence meaningful residue analysis is difficult to obtain. Studies on cereals and cereal products by Winteringham (11), Bridges (12) and Winteringham et al (13) concluded that in cereals, methylation mainly of nitrogen - and sulfur - containing groups in the proteins accounts for a large part of the decomposition of sorbed fumigant. Shrader, Beshgestoor and Stenger (21) had earlier reached the same conclusions from studies of the reaction of methyl bromide with sulfur - and nitrogen - containing compounds which are known to be present in cereals. Hydrolysis or hydration has also been considered as a possible mode of decomposition of methyl bromide (24). This has been shown by Winteringham et al (13) and Scudamore and Heuser (23) to be incorrect. It seems probable that the protein fraction of the fumigated wheat is mainly responsible for the incorporation of methyl bromide.

The object of the investigation is to study the nature and extent of the reaction between methyl bromide and the proteins of cocoa beans.

CHAPTER II

EXPERIMENTAL

A. APPARATUS AND MATERIALS

1) Instruments

Amino Acid Analyzer

Amino Acid analyses were carried out on a Beckman Spinco model 120C Amino Acid Analyzer using the procedure (manual AT B 033) for the 4-hr analysis.

2) Liquid Scintillation Counter

A Nuclear-Chicago corporation (Chicago, Ill.) Unilux II Liquid Scintillation counting system with an external standard Ba^{133} equipped with a model 8912 Electronic Calculator (Nuclear Chicago Corp) and a model 8473 IBM Electric Typewriter was employed for all liquid scintillation counting of radioactive substances. Discriminators and attenuators for each channel were adjusted with the aid of Nuclear-Chicago quenched carbon-14 standard sets (model 180060). Plot of percent efficiency for ^{14}C versus channel ratio was prepared. A minimum of 10,000 counts per sample were accumulated, gross counts were transformed to net counts per minute and this was converted to net disintegrations per minute per gram of protein or similar units.

3) Gas Chromatography

Beckman GC-45 dual column gas chromatograph equipped with flame ionization detector and with an in-

tegrator as part of the recording system was used for analyzing the sugar fraction. The sugars were analyzed as their trimethylsilyl (TMS) ether derivatives (25-27).

4) Thin Layer Chromatography

Thin layer chromatography analyses were carried out on Eastman Chromatogram silica gel plates in two solvent systems: n-butanol: acetone: diethylamine: water (10: 10: 2: 5); chloroform: methanol: 17% ammonia (2: 2: 1). Detection was achieved by spraying the plates with ninhydrin, iodine (and viewing with ultraviolet light), Pauly reagent (diazosulfanilic acid) (26, 28, 30) and the Sakaguchi reagent (28, 29).

5) Chemicals

Synthesis of Methylated Derivatives of Histidine, Lysine and Arginine

Nϵ- monomethyl-L-lysine, 7-N - monomethyl-L arginine and 1 - and 3 - N - methyl-L-histidine were prepared by a modified method of Bridges (12).
N -α- acetyl derivatives of L-lysine, L-arginine and L-histidine (100 mg) were placed in 0.5 x 20 cm tubes. By pipette 0.9 ml deionized distilled water and 200 mg of methyl iodide were added. The tubes were cooled in dry-ice acetone mixture and sealed. Methylation was affected by heating the tubes and their content at 90° ±1° in an oil bath for 7 days. Excess solvent and

methyl iodide were removed on rotary evaporator. The contents were refluxed for 1 hour in 2N HCl (25 ml) at 110°± 1° to remove the O-acetyl group. Excess acid was removed by repeated evaporation. The methylated products were dissolved in 0.1M acetic acid solution. The solutions were treated with charcoal and Amberlite IR 120 (hydrogen form). After stirring for 1 hour the resin was filtered off, washed thoroughly with de-ionized distilled water and stirred for 15 min in 5M ammonium hydroxide solution (50 ml). The mixtures were filtered and the filtrates were concentrated and placed separately on preparative thin layer chromatography (t.l.c.) plates (1.0 mm) (chloroform: methanol: 17% ammonia; 2: 2: 1). The desired monomethylated band was located, by spraying a small strip of the plate with ninhydrin, and scraped from the plates. The silica gel containing the methylated derivative was placed in a small column and eluted with 0.1 M acetic acid solution. The samples were characterized by thin layer chromatography and paper chromatography using the solvents: chloroform: methanol: 17% ammonia (2: 2: 1); n-butanol: acetone: diethylamine: water (10: 10: 2: 5) and pyridine: acetone: 3 M ammonium hydroxide (50: 40: 20). Staining (26, 28) of the tlc plates and the paper chromatograms first with ninhydrin and then with iodine followed by viewing with ultraviolet light, Pauly reagent and/or the Sakaguchi reagent was

employed for detection of the different methylated amino acid products.

^{14}C methyl bromide and Aquasol were obtained from New England Corp. Tri-sil Z, TMS -α- D-Glucose, TMS-β-D-Glucose, TMS-L-Arabinose ,TMS-D-Galactose, TMS-D-Xylose,TMS-D-Mannose, TMS-Lactose were from Pierce Chemical Company. Hyamine Hydroxide is a Rohmn and Haas Inc. chemical. Commercially fumigated (with methyl bromide) and unfumigated cocoa beans were received from Mr. Robert Ofori of the Government Chemical Laboratory, Accra, Ghana. Other materials and chemicals were reagent grade.

B. PROCEDURES

1) Separation of Fats, Alcohol-Insoluble and Alcohol-Soluble Proteins and Free Amino Acids

Fractionation of the material was performed as outlined in scheme 1.

The shells were removed from the nibs of a 50 g sample of whole cocoa beans taken from a 3 kg sample. Both parts were ground separately in a Wiley Mill to pass first through a 0.841 mm mesh sieve and later through a 0.595 mm mesh sieve. The finely ground material (shells or nibs, 25g) and 10 mg Norleucine (internal standard) were extracted in a soxhlet extractor with light petroleum ether (30° to 60°C) for 5 hrs. The petroleum ether was evaporated and the fat-containing residue analysed.

Five-gram portions of the material which had been extracted with petroleum ether were placed in centrifuge bottles, extracted with 30 ml portions of ethanol: water (70: 30) solution for 1 min and centrifuged at 500 rev/min for 10 min. This extraction was repeated until the supernatant gave no ninhydrin reaction. The residue was dried and analysed as alcohol-insoluble proteins.

The combined ethanol solutions were evaporated to dryness on a rotary evaporator and the residue was redissolved in deionized water. The aqueous suspension was centrifuged and the residue, therefrom, retained

for analysis as alcohol-soluble protein. The aqueous supernatant from the above suspension contained the free amino acids of the cocoa beans. These amino acids were further purified by ion-exchange chromatography (0.9 x 20 cm Amberlite IR-120 (H^+ form)). After placing the supernatant on the column, the column was washed with 50 ml of a 20% solution of isopropanol, then with a 25 ml of a 50% solution of isopropanol and finally with 200 ml of deionized water. This procedure removes flavonoids (24). The amino acids were eluted from the column with 150 ml of 3N-ammonium hydroxide solution (31). The effluent was evaporated to dryness. The residue was redissolved in deionized water and again taken to dryness. This was repeated three times and then the residue was finally dissolved in an 0.2N sodium citrate buffer, pH 2.2 and analyzed for the free amino acids.

The alcohol-soluble and the alcohol-insoluble protein residues were hydrolyzed by treatment with 6N hydrochloric acid at $110^\circ \pm 1^\circ$ for 12 hr, 18 hr, 24 hr, and 48 hr (32). The protein hydrolysates were also purified by ion-exchange chromatography (0.9 x 20 cm Amberlite IR-120 (H^+ form)).

2) Extraction of Sugars

The sugar fraction of the cocoa bean was obtained from both the shells and the nibs. The ground, petroleum ether extracted material (shell or nibs, 10 g) was pulverised for 5 min with 150 ml of a 20% methanol solution (33) in a Waring Blender. The suspension was centrifuged for 20 min at 2000 rev/min, the supernatant was decanted and made alkaline (pH 10) using 1N potassium hydroxide solution. The polyphenols were precipitated with a saturated lead acetate solution (25 ml) and then centrifuged for 15 min at 2000 rev/min to remove the phenol-lead complex. The clear supernatant contains the sugars. The sugars were further purified by ion-exchange chromatography (0.9 x 20 cm Amberlite IR - 120 (sodium form)) and (0.9 x 20 cm Dowex 2 - X8 (chloride form)). The sodium form of the cation resin was necessary to prevent sucrose hydrolysis on the column (33).

The eluant from the ion-exchange column was freeze-dried. The sugar-containing residue was redissolved in deionized water and again freeze-dried. The sugars were extracted into pyridine (stored over potasium hydroxide pellets) by warming in a water-bath at 60° for 15 min. By pippete 1 ml of "TRI-SilZ" (N- (trimethylsilyl) - imidazole)in pyridine was added to the sample in a small bottle and shaken vigourously for 30 sec. This was allowed to stand 30

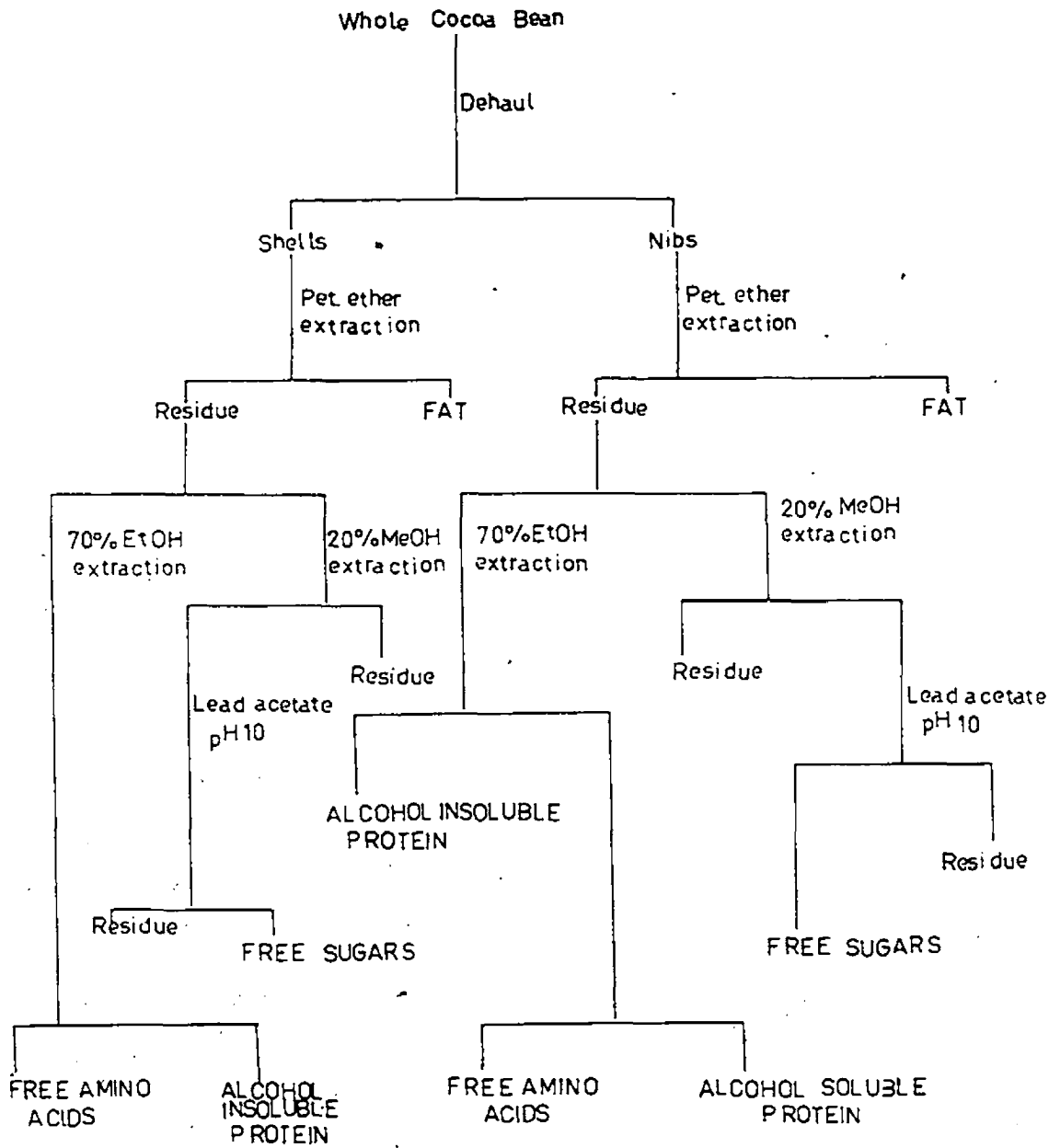
min with occasional shaking, and analyzed for the free sugars.

3) Treatment of Whole Cocoa Bean and Cocoa Powder With ^{14}C - Methyl Bromide

The exposure of the cocoa bean parts to ^{14}C-methyl bromide was conducted in a modified Winteringham et al (35) apparatus as shown in Figure 1. This consists of a sample flask (S) (350 ml) and a gas flask (G) (350 ml) connected by a communication tube with a stopcock.

Whole cocoa beans (50 g) with 6.9% moisture content were introduced into the sample flask (S) and an ampoule of ^{14}C-methyl bromide was introduced into the gas flask (G). The flasks were evacuated, the stopcock closed and unlabelled methyl bromide added to the gas flask (G). The ampoules were broken after which the two flasks were joined by opening the stopcock. The cocoa beans were exposed with stirring to an average concentration of 250 mg of methyl bromide per liter for 48 hr at 25° . The average specific activity of the ^{14}C-methyl bromide was 0.14 μCi/mg.

In a second experiment finely ground nibs (50 g) were exposed to the same average concentration of 250 mg of methyl bromide per liter and average specific activity of 0.14 μCi/mg. The ^{14}C-methyl bromide treated materials were fractionated as outlined in Scheme 1.



Scheme 1: Flow Diagram for Sample Preparation

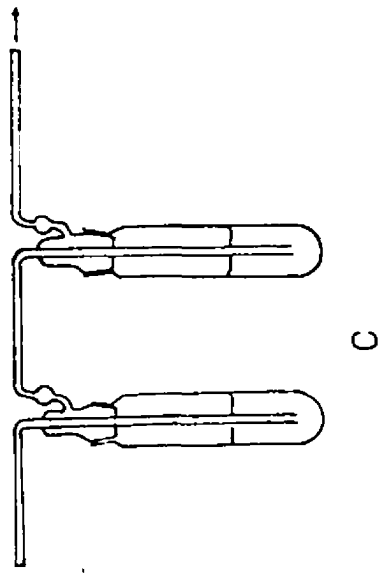
Fig. 1. Apparatus for ^{14}C-Methyl Bromide fumigation of Cocoa Beans.

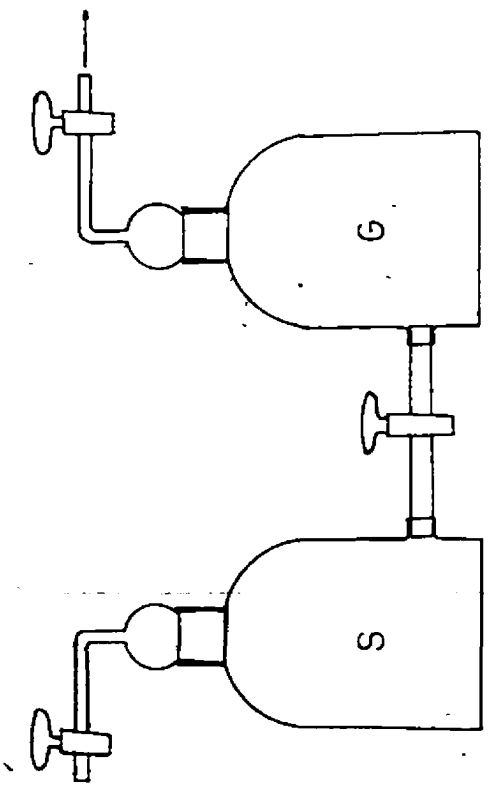
S ; sample flask.

G ; gas flask.

C ; bubble tubes, each tube contains 10ml of monoethanolamine + ethylene glycol monoethylether (1:1 v/v)

Figure 1





C. METHODS OF ANALYSIS

1) Amino Acid Analyses

Analyses were carried out with a Beckman 120C Amino Acid Analyzer operated at high sensitivity. The standard calibration mixture was prepared by diluting Beckman Amino Acid calibration mixture Type I with 0.2 N sodium citrate buffer, pH 2.2. A separate calibration mixture consisting of the diluted Beckman Type I mixture plus hydroxylysine, hydroxyproline and S-methyl cysteine, at concentrations of 0.4 μmole per ml, in 0.2N sodium citrate, pH 2.2 was also prepared.

All samples were applied to the columns with Beckman manual sample injectors having a 200 μl sample loop. Chromatograms were carried out on a 0.9 x 69 cm column packed with Beckman PA-28 resin (with resin bed 56 cm) and a 0.9 x 23 cm column packed with Beckman PA-35 resin (with resin bed 5.5 cm). The starting buffer and temperature for the 'long column' were 0.2N sodium citrate, pH 3.25 ± 0.02 and 55.5°, respectively. At 95 min the buffer was changed automatically to 0.2N sodium citrate, pH 4.25± 0.02 with no change in temperature. A single buffer system, 0.35N sodium citrate, pH 5.25± 0.02 was employed for the 'short column'. The temperature was 55.5°. The flow rate for both columns was 68 ml/hr.

A Radiometer (Radiometer A/S, Copenhagen NV, Denmark) was used for all pH measurements.

After every analysis the columns were regenerated with 0.2N sodium hydroxide solution and equilibrated with the appropriate starting buffer.

2) Analyses of Radioactive Substances

Radioactivity was determined using a Nuclear-Chicago Liquid Scintillation counter Unilux II. In general 1 ml of the solution to be counted was pipetted into a vial and 10 ml of Aquasol added. The vial was shaken and the mixture counted.

Dry alcohol-insoluble proteins (100 mg) were first digested in 1 ml of 0.3N potassium hydroxide solution at 37° for 1 hr. Then 1 ml of Hyamine hydroxide (p - (disobutylcresoethyl-ethoxyethel) - dimethyl-benzenyl ammonium chloride) was added to effect complete dissolution of the digest. The alcohol-soluble proteins (100 mg) and the sugar fraction (100 mg) were dissolved directly in 1 ml Hyamine hydroxide with gentle heating. All solutions were bleached with a trace of hydrogen peroxide before counting (37).

3) Analyses of Sugar Fraction

The total reducing sugar content of the shells and the nibs was determined spectrophotometrically by the phenol-sulfuric acid method of Dubois et al (38). The sugar fraction was resolved using a Beckman GC - 45 gas chromatograph. Each of the various standard TMS-sugars (100 mg/ml) in hexane were accurately pipetted (0.5 ml) into 10 ml volumetric flasks and diluted to

10 ml with n-hexane. A 1 ml aliquot was injected into the gas chromatograph. The retention time and the area of each of the standards were recorded. The calibration mixture was prepared by pipetting 0.5 ml of standard TMS-sugars into 10 ml volumetric flask and diluting to 10 ml with n-hexane.

The sugars were assayed under the conditions given in the legend to Figure 3.

4) Bromide and Nitrogen Determination

The residual total bromide was determined by the method of Turner (11). The total protein nitrogen was determined by Kjeldahl method (39).

CHAPTER III

RESULTS

1) Total Bromide Content

Both fumigated and unfumigated cocoa beans were analyzed for total bromide to ascertain in what portion of the bean methyl bromide decomposed. The shells and nibs were ground separately and extracted with petroleum ether. The fat-containing portion of the bean appears to retain less than 1 part /million of bromide residue (Table I). Fumigated beans contained 15.4 parts/million bromide in the nibs protein and 46.5 parts/million bromide in the shell protein. There was a surprisingly high content of inorganic bromide in the unfumigated bean protein, where the nibs contained 6.65 parts/million bromide and the shells 9.89 parts/million bromide.

2) Amino Acid Composition

Since the main area of methyl bromide decomposition appears to be in the protein portion of the beans, a study of the amino acid content of these proteins was undertaken. In Figure 2 is a typical amino acid analysis spectrum of alcohol-insoluble protein from nibs treated with methyl bromide. The spectra for alcohol-soluble proteins of nibs, alcohol-insoluble proteins of the shells and free-amino acids of nibs and shells are qualitatively the same as that of the

alcohol-insoluble proteins. The amino acids were identified and quantified by comparison with a set of standard amino acids. Peaks I, II, III and IV could not be identified. When comparing proteins obtained from fumigated and unfumigated beans, on the long column, the spectra were again qualitatively the same except that peak II was present only in fumigated samples. Samples were chromatogrammed on the short column with one buffer system and different temperatures, 28°, 30°, 35°, 40° and 55.5°. Since the separation of the basic amino acids and their methylated derivatives is very sensitive to pH, two buffer systems, 0.38N sodium citrate, pH 4.25± 0.02 and 0.35 N sodium citrate, pH 5.36 ± 0.02 were employed in combination with the different temperatures for their separation. Separation of the methylated amino acids from their unmethylated precursors could not be achieved.

A qualitative study of the amino acids in cocoa beans indicated that these amino acids are fairly evenly distributed throughout the bean with the exception of cysteine which is concentrated in the nibs (Table II). Tryptophan, glutamine and asparagine could only be detected in the free amino acid fraction, since all three are decomposed by acid hydrolysis of the protein fractions. Glutamine and asparagine could not be completely resolved.

TABLE I: Total Bromide and Nitrogen Content of Cocoa Beans

Moisture Content %	Nitrogen Content %	Total Reducing Sugars (% by wt.)		Total Residue Bromide (PPM)					
				Fumigated			Unfumigated		
		Shell	Nib	Fat	Nib	Shell	Fat	Nib	Shell
6.9	22.2	1.0	2.3	<1.0	15.4	46.5	<1.0	6.65	9.89

Fig.2. Amino Acid Composition of Alcohol-Insoluble Proteins of Cocoa Bean Nibs.

FIGURE 2

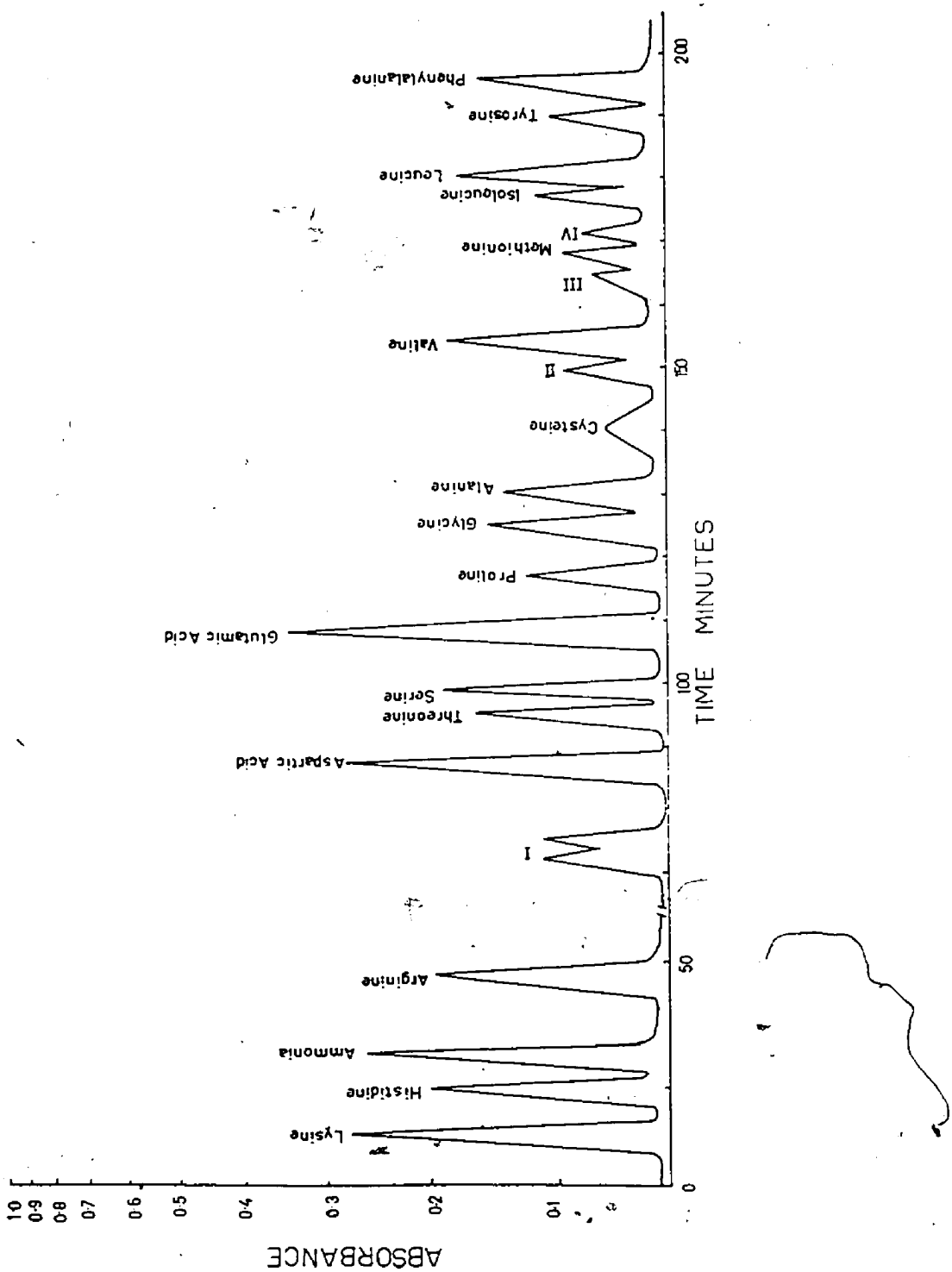


TABLE II: Composition of Cocoa Beans

Amino Acid	Free Amino Acids (mg/100g)		Alcohol-Soluble Protein (mg/100g)	Alcohol-Insoluble Protein (mg/100g)	
	Shells	Nibs	Nibs	Shells	Nibs
Lysine	33.1	44.8	10.7	112	337
Histidine	6.0	10.1	3.5	21	67
Arginine	19.8	39.7	14.6	41	228
Aspartic Acid	18.4	23.0	15.7	835	2350
Threonine	19.7	23.9	5.2	310	1000
Serine	36.5	43.5	8.0	379	1525
Glutamic Acid	75.1	91.0	23.7	1283	3946
Proline	23.5	28.4	8.3	712	1082
Glycine	7.4	8.9	7.7	459	1462
Alanine	34.7	46.8	6.1	479	905
Cysteine	-	-	-	-	363
Methionine	Trace	Trace	Trace	Trace	Trace
Valine	33.7	40.9	8.1	633	1281
Isoleucine	22.9	25.1	5.8	422	900
Leucine	49.0	75.2	10.9	701	1639
Tyrosine	31.0	41.6	5.5	113	336
Phenylalanine	42.9	61.3	10.1	429	1335
Tryptophan	21.3	-	-	-	-
Glutamine	Trace	-	-	-	-
Asparagine	Trace	-	-	-	-
β-Alanine	Trace	Trace	-	-	-

3) Reproducibility of the Extraction Method

A large number of extractions and manipulations are necessary for preparation of the cocoa bean extracts. These extractions might introduce possible sources of error. The deviation of results for total amino acid content between duplicate extracts of the same sample was never larger than 2%.

4) Sugar Fraction

Figure 3 shows the gas chromatogram of the sugar fraction from ^{14}C-methyl bromide treated nibs. The chromatograms for the commercially fumigated and unfumigated sugar fractions of the nibs were qualitatively the same as that of the laboratory fumigated nib sugar fraction except for peak I which appeared only in the ^{14}C-methyl bromide treated sample. The following compounds were identified (Fig. 3); α- and β-glucose, arabinose and/or xylose, α-fructose and/or mannose, galactose, three unknowns.

5) ^{14}C-Methyl Bromide Studies

The amount of radioactivity incorporated into the various protein fractions of the shell and nibs is shown in Table III. Most of the methyl bromide was decomposed by the shell proteins but an appreciable amount (ca 32.1%) also reached the nibs.

As the nibs are the portion of the bean consumed by humans, powdered nibs were exposed to ^{14}C-methyl bromide (Table III) in order to obtain enough methylated amino acids for analysis. The alcohol-insoluble protein was then hydrolyzed. Then 0.75 ml of the hydrolyzed solution was pipetted onto the short column of the amino acid analyzer and for 10 min a fraction was collected directly from the column which contained the acidic and neutral amino acids. The basic amino acids were then collected. Both solutions were concentrated to 1 ml and counted.

The basic amino acid fraction was desalted (40) and was subjected to thin-layer chromatography together with standard methylated basic amino acids (Table IV). With thin-layer chromatography analysis of the basic amino acids obtained from commercially fumigated beans a faint trace of methlated basic amino acid could be detected. (R_f 0.49: solvent 1 and R_f 0.63: solvent 2). No methylated basic amino acids were detectable in unfumigated beans.

Fig. 3: Sugar Profile of Cocoa Beans.

Column: 6in x 2mm glass, 3% OV 225 on Chromosorb W-HP, 100-120 mesh.

Column Temperature: Programmed; 140°-240°C/min.

Detector: F.I.D.

Flow rate(helium) ;25cc/min.

FIGURE 3

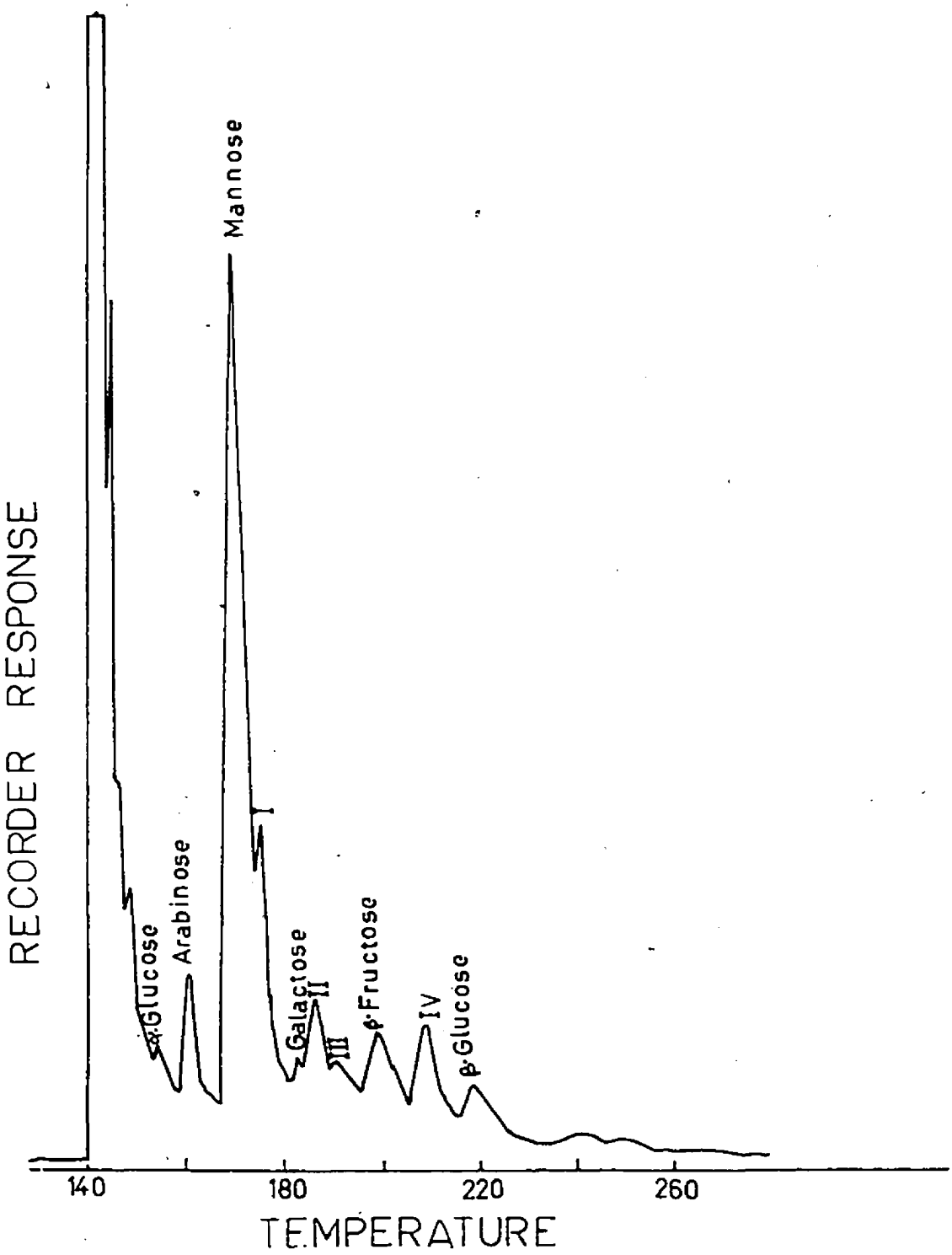


TABLE III: Incorporation of Carbon 14 into Cocoa Beans

Sample	Alcohol-Soluble Protein Fraction (dpm/g)	Alcohol-Insoluble Protein Fraction (dpm/g)	Basic Amino Acid Fraction[c] (dpm/g)	Neutral and Acidic Amino Acid Fraction[c] (dpm/g)	Sugar Fraction (dpm/g)
Shells[a]	975	18000	-	-	-
Nibs[a]	202	9250	-	-	-
Nibs[b]	760	29000	4300	12600	168

[a] From fumigation of whole cocoa beans

[b] From fumigation of powdered cocoa nibs; ground to pass through a 0.595 mm mesh sieve.

[c] From the alcohol-insoluble protein.

TABLE IV: R_f Values of Standard Amino Acids and Unknowns From Cocoa Beans (Thin Layer Chromatography)

Amino Acid	Solvent 1[a]	Solvent 2[b]
	R_f	R_f
Standards		
Lysine	0.37	0.50
N -Methyl lysine	0.17	0.46
Histidine	0.62	0.55
1-N-Methyl histidine	0.59	0.52
Arginine	0.12	0.24
7-N-Methyl arginine	0.12	0.23
Methyl cysteine	0.64	0.68
COCOA BEAN UNKNOWNS		
I	0.16 (3)[c]	0.47
II	0.50 (73)[c]	0.63
III	0.59 (23)[c]	0.53

[a] Butanol /acetone/diethylamine/water (10/10/2/5).

[b] Chloroform/methanol/17% ammonia (2/2/1).

[c] Approximate percentage of radioactivity associated with a particular zone.

CHAPTER IV

DISCUSSION

The breakdown of methyl bromide during fumigation occurs mainly in the shells. Determination of the residual bromide content in nibs and shells shows that the shells absorb about 3 times more of the fumigant than the nibs. The surprisingly high content of inorganic bromide in the unfumigated cocoa bean protein (Table I) may have occurred as a result of uptake of bromide from the soil, predominantly in water-soluble ionic form (41). The bromide ion in the soil may be present "naturally" or may result from the breakdown of bromine-containing chemicals.

The fate of the methyl group of methyl bromide is qualitatively the same as that of the bromide group, that is, a much higher percentage of the methyl group is retained in the shell than in the nibs (Table III). Most of the decomposition of the fumigant takes place in the alcohol-insoluble proteins of both the shells and nibs. Sugars appear to contribute little to the breakdown of the fumigant (Table III). The greater breakdown of methyl bromide in the shells is probably a result of the fumigant coming into contact with that portion of the cocoa bean first.

The amino acid composition of the free amino acids, alcohol-soluble proteins and alcohol-insoluble proteins are qualitatively the same except for cysteine which is concentrated in the alcohol-insoluble proteins (Table II). With the exception of methionine which occurs in trace amounts, the essential amino acids occurred in substantial amounts. Schormuller and Winter (34) noted that methionine was the limiting amino acid of Accra (Ghana) cocoa beans. The alcohol-insoluble proteins make up the major portion of the amino acid-containing material in cocoa beans (Table II). The greater concentration of alcohol-insoluble proteins probably explains why methyl bromide decomposition primarily takes place in this portion of the bean protein. The only difference between the amino acid analysis of the fumigated and unfumigated beans was a peak appearing between cysteine and valine in the amino acids of the fumigated sample (Figure 2).

The amino acids from the alcohol-insoluble nib proteins were divided into a basic amino acid fraction (histidine, lysine and arginine) and a fraction containing the neutral and acidic amino acids. The incorporation of radioactivity into the neutral and acidic amino acid fraction was three times greater than into the basic amino acid fraction (Table III). The major site of methyl bromide decomposi-

tion in wheat is reported to be in the acidic amino acids (6). The methylation of the acidic and neutral amino acids is probably via a nucleophilic attack of the α-nitrogen of the amino acid on the methyl bromide. The bromide is displaced and an N^{α}-methyl amino acid results as a product. The amino acids methylated in this manner are necessarily at the N-terminal end of the protein. From a purely statistical basis one would expect that the methylation of the acidic and neutral amino acids would predominate over that of the basic amino acids by a factor of 38 (mol of acidics and neutrals/mol of basics = 38 for the alcohol-insoluble proteins of nibs) rather than by the observed factor of 3. The basic amino acids seem to be more reactive than expected.

The basic amino acids were further analyzed by thin-layer chromatography. Three radioactive spots were obtained. Comparison of these spots with methylated basic amino acids indicated that 1 - or 3-monomethyl histidine and N^{ϵ}-monomethyl lysine accounted for two of the radioactive spots. The other spot may arise from 1, 3-dimethyl histidine but was not identified. Bridges (12) has shown through combined radioactive tracer-chromatographic techniques that the principal reaction between ^{14}C-methyl bromide and nitrogen-containing groups of wheat protein was with the imidazole nitrogens of histidine and the ϵ-

nitrogen of lysine.

The sugar profile of unfumigated, commercially fumigated and ^{14}C-methyl bromide fumigated nibs was qualitatively the same except for a peak (peak I) which appeared in the sugar profile of ^{14}C-methyl bromide fumigated nibs (Fig. 3).

This study on the site of decomposition of methyl bromide in cocoa beans agrees with studies on the decomposition of the fumigant in wheat (6,11-13). The methyl group of methyl bromide is bonded to α - nitrogen of amino acids and to the basic amino acids at the 1 - or 3-nitrogen of histidine and the N^{ϵ} - position of lysine. In addition most of the methyl bromide is decomposed in the shell of the cocoa bean.

Winteringham considered the possible toxicological and nutritional significance of fumigating wheat with methyl bromide and showed through metabolic studies of the principal end-products of fumigation that the principal fumigant decomposition products are neither toxic nor that their formation would be associated with any significant reduction in essential food constituents (11). N^{ϵ}-methyl lysine has been shown to be active in the amino acid dependent adenosine triphosphate exchange reaction by amino-acyladenylate synthetase from calf liver. However, the K_m value was approximately 10,000 times that of L-lysine (42). The amount of reaction occurring un-

der typical fumigation conditions of cocoa beans is so small that loss of the semi-essential amino acid, histidine and the essential amino acid lysine are negligible.

CHAPTER V

SUMMARY AND CONCLUSION

Methyl bromide absorbed by cocoa beans under the conditions of fumigation undergoes chemical decomposition with formation of inorganic bromide and a series of methylated derivatives. The main site of decomposition of methyl bromide is in the alcohol-insoluble proteins of the shells but an appreciable amount (ca 32%) reached the nibs.

The basic amino acids seem to be more reactive than the neutral and acidic amino acids. Three methylated products occur in the basic amino acid fraction of the alcohol-insoluble proteins of methyl bromide treated nibs. These have been identified as 1 - or 3-monomethylhistidine, and N-ϵ-monomethyllysine; the third product may arise from 1, 3-dimethylhistidine.

There is no appreciable loss of any of the essential amino acids in cocoa proteins by N-methylation when cocoa beans are fumigated with methyl bromide under normal conditions.

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PART II

FIELD DESORPTION MASS SPECTROMETRY OF AMINO ACIDS AND PEPTIDES

CHAPTER I

INTRODUCTION

Chemical and/or enzymatic cleavage of protein chains into oligopeptide fragments and the separation of the oligopeptides presents no fundamental difficulties (1,2). However, a rapid, unequivocal and straightforward determination of the amino acid sequence in the oligopeptides thus obtained is not always possible, the more so since in most cases only micro- amounts of material are available. All the "wet chemical methods" used to this end are extremely laborious and usually permit determination of only a partial sequence. However, as the problem of primary structure determination in peptides and proteins is so crucial and the cases encountered are so diverse, it is advantageous to have several methods of determination to check and supplement one another.

Mass spectrometry has been recognized (3,4,5) as a potentially promising technique for the determination of the sequence of amino acids because of the great savings in time, ease of obtaining spectra of an entire oligopeptide and of the small amounts of material necessary. From a chemical point of view the mass spectrometer seems, in principle, an ideal method to apply to peptides and proteins since:

1. peptides have a linear structure (cyclic ones (5-9) and depsipeptides (10-16) can often be opened specifically to linear ones);
2. the backbone consists of identical repeating units (-NH - CH - CO -) containing bonds expected to cleave easily under electron impact;
3. the structural differences are due to the substituents (R) attached to the - CH - groups in the backbone of the peptide (I).

$$^{+}NH_3 - \overset{R_1}{\underset{|}{CH}} - CO - NH - \overset{R_2}{\underset{|}{CH}} \ldots\ldots\ldots NH - \overset{R_n}{\underset{|}{CH}} - COO^{-}$$

I

The major obstacle to the general application of mass spectrometric technique to sequencing of amino acid residues in oligopeptides is the problem of volatility (17,18). Due to their poor volatility from intermolecular hydrogen bonding and to their susceptibility for thermal decomposition (19), free peptides of longer than two amino acids are not suitable for investigation in the electron impact mode of the spectrometer (18,20).

The relatively low volatility and thermal instability of peptides have led to the development of many chemical derivatization techniques (21-34). Acylation of

the amino-terminal amine function with the N-protection groups such as acetyl (6), trifluoroacetyl (35,36), heptafluorobutyl (37), benzyloxycarbonyl (38,39), phthaloyl(40), ethoxycarbonyl (41,44), hexanoyl (42), 2,4-dinitrophenyl (43), decanoyl (45), heptadecanoyl (38), octadecanoyl (38), stearoyl (38), benzoyl (46), methoxycarbonyl (47) and phenylaminocarbonyl (47) have been reported. Alpin et al (47), compared the utility of the various N-protecting groups and showed that the acetyl derivative provides the best combination of volatility and abundance of sequence ions. Esterification of the carboxyl terminal carbonyl function with methyl (4,21,41), ethyl (21), and t-butyl (22) groups has been examined. Permethylation of peptides employing methyl iodide in dimethylformamide in the presence of silver oxide (48-50); sodium hydride in dimethyl-sulfoxide and methyl iodide (51,52,33); sodium hydride in dimethylformamide and methyl iodide (55,56); sodium hydride in dimethyl-acetamide and methyl iodide (54) has been studied. The permethylation further reduces hydrogen bonding and increases volatility. Comparative studies of the different N-permethylation techniques showed (57) that the use of silver oxide frequently resulted in undesirable side reactions, particularly for peptides containing aspartic acid, glutamic acid, methionine or tryptophan residues (58). With sodium hydride in dimethyl formamide, C-methylation of aspartic acid, glutamic acid and glycine has been observed (59). Sodium derivatives of dimethylsulfoxide and dimethylacetamide were much less

harmful, but even those could entail complications (59). The significant difference between all methylation techniques is not the solvent but rather the base which is employed (57). Prior modication of such amino acids as arginine (32), methionine (32),cystine(22), cysteine (22), lysine (33) and ornithine (33), is essential in any permethylation technique.

Reduction of N-trifluoroacetyl, N-pentafluoro-propionyl and N-heptafluorobutyryl oligopeptide methyl esters by lithium aluminium deuteride and the subsequent O-trimethylsilyl-ation has been reported to yield the most volatile peptide derivatives known(30). All naturally occurring amino acid residues, including arginine, histidine, tryptophan, glutamine and asparagine, can be derivatized by this technique without previous modification(30) of these amino acids.

A general, simple and quantitative derivatization technique suitable for oligopeptides at the 1-10 nmole level has not yet been developed (60).

Other ionization methods used for amino acid sequence determination are field ionization (f.i.) and chemical ionization (c.i.). In the case of field ionization, reports by Brown et al (61) and Winkler et al (62) demonstrate that this technique gives a molecular ion of enhanced intensity compare to e.i. mass spectrum. On the other hand often the f.i. mass spectra alone do not permit the derivation of the complete sequence. In recent reports Fales et al (28),

Gray et al (26) and Milne et al (65) demonstrated the application of c.i. in peptide sequence analysis, in each case there was increased quasi-molecular ion $[M+1]^+$ intensities relative to the e.i. mass spectra. The cleavage of peptide bonds was shown to occur in two ways (at the amino-terminal acyl carbonium ion and at the complementary carboxyl terminal iminium ion) and the determination of the amino acid sequence of the peptides was possible.

The possibilities of combining e.i., c.i. or f.i. with collisional activation for peptide sequencing have been demonstrated (66-68). Orlov et al (69,70,71) have reported photoionization spectra of some derivatized oligopeptides. The ionization of peptides by single ion impact in a tandem mass spectrometer has been investigated and the importance of using evaporation of the oligopeptide from inert surfaces such as a Teflon probe introduced directly into the source of a mass spectrometer was emphasized. Volatility, when this technique was used, was considerably enhanced (72 - 75).

Several groups (53,76-80,84,92) have reported on their attempts to sequence individual oligopeptide chains in unseparated mixtures of oligopeptides such as might be obtained by proteolytic digestion of a protein fragment. All the groups emphasize the necessity in this connection of partial vaporization studies; the changes in relative abundance of peaks at varying temperatures serving to distinguish which peaks arise from which oligopeptide.

Sutherland et al (81) and other workers (82,83) have recently proposed a promising scheme for polypeptide sequencing involving the dipeptidylaminopeptidase degradation of peptides to mixtures of dipeptides whose derivatives can be separated and analyzed by gas chromatography-mass spectrometry.

Recent studies exploiting the gentle ionization features of chemical ionization have demonstrated that it is possible to evaporate many underivatized dipeptides and obtain spectra useful for identification and sequencing (85). McLafferty et al (86) have developed a chemical ionization technique that provides for direct introduction of liquids containing dissolved underivatized peptides into a chemical ionization source. This technique has made possible the observation of protonated parent molecule ions of a variety of peptides including Ala. Ala. Ala. His and hexaalanine (87,88).

The volatility problem exists primarily because of the instability of peptide molecules at temperatures required for their evaporation. Energy required to break bonds attaching peptides to a solid surface is distributed among internal degrees of freedom and absorbed in decomposition reactions. From the standpoint of structure determination mass fragments still provide much useful information. Nevertheless it is desirable to minimize fragmentation if mass spectra are to be correlated with neutral molecule structures.

Field desorption deposits energy almost exclusively in bonds holding the molecules to the surface (89). The success of field desorption in minimizing the equilibration of energy among internal degrees of freedom of complex molecules points up the kinetic nature of the evaporation process which takes place at low pressures in a a mass spectrometer ion source (74). The utility of field desorption mass spectrometry in the study of organic molecules which are labile and/or have low volatility, has been demonstrated (89-91,93-106,110-114, 118-122).

In field desorption mass spectrometry a substance is deposited (by concentration of a solution) on an emitter comprising a tungsten wire (10um diameter) on which semi-conducting organic microneedles are grown (93,107-109). In the ion source at about 10^{-6} Torr the wire is given a high positive potential; when the emitter is positively charged the strong electrostatic field results in the formation and removal of ions of "impurities" adsorbed on the surface of the emitter. This process is basically an evaporation of positive ions over a potential barrier on the surface due to the strong electric field. Loss of electrons from the adsorbed substance occurs by tunnelling. Frequently the emitter is simultaneously heated by an electric current of several milliamperes (89). Indirect heating of the emitter by infra red radiation has been applied by Winkler et al (118).

Winkler and Beckey (62,119) have established the utility of field desorption in studies of oligopeptide structure.

The field desorption mass spectra of an underivatized pentapeptide and an underivatized nonapeptide, Arg. Gly_3. Pro.Gly_3.Arg have been reported to show intense molecular ions but fragmentation of the oligopeptides was insufficient to allow sequence determination.

Williams *et al* (120) have used field desorption mass spectrometry for the determination of the molecular weight of the peptide antibiotic Echinomycin (mol.wt. 1100.4); only a molecular ion peak was obtained in the high-mass region. A complete spectrum of echinomycin was obtained using a modified electron impact technique in which the sample was evaporated close to the electron beam. A combination of field desorption and electron impact mass spectrometry have been used by Rapp *et al* (121) to sequence both derivatized and underivatized peptides including an octapeptide.

The object of the present investigation is to apply field desorption mass spectrometry to the sequencing of underivatized oligopeptides. A number of parameters such as emitter heating currents, diverse ions and mixtures are studied.

CHAPTER II

EXPERIMENTAL

A. APPARATUS AND MATERIALS

Instrumental

The mass spectrometer used was a Varian MAT model CH5 DF with combined f.d.-f.i.-e.i. source. The anodes were of 10 μm diameter tungsten wire spot welded on supporting posts mounted on to ceramic holders and conditioned with benzonitrile in a Varian apparatus in a manner similar to that described by Beckey (89).

Chemicals and Materials

1) The following amino acids and peptides were obtained from Sigma Chemical Co. H.Lys.OH.HCl, H.Phe.OH, H.Thr.OH, H.Leu.OH, H.Tyr.OH, H.Trp.OH, H.His.OH, H.Phe.Val.OH, H.Gly.Ile.OH, H.His.Tyr.OH, H.His.Ser.OH, H.His.Lys.OH.HBr, H.Met.Ala.Ser.OH, H.Pro.Phe.Gly.Lys.OH.$COCF_3$, H.Trp.Met.Asp.Phe.NH_2, NCBZ.Pro.Gly.Leu.Gly.Pro.OH, Bradykinin. H.Pro.Leu.Gly.NH_2 was from Beckman Bioproducts Dept. Glutathione (oxidized and reduced forms) was obtained from Calbiochem.

2) Ethanol, Acetic Acid, Acetic Anhydride ACS grades were used.

3) Ethanol-d_6 was from Merck Sharp & Dohme Ltd. Acetic Anhydride-d_6 and deuterium oxide were from Stohler Isotope Chemicals.

4) Distilled water was deionized by passing through a column of mixed-bed ion-exchange resins obtained

from Barnstead Still and Sterilizer Co. Boston, Massachusetts (Model BD-1).

B. PROCEDURES

1) General Procedure

The amino acid and peptide samples (0.2 mg) were prepared as either 75% ethanol or 0.1 M Acetic acid solutions (100 µl) and transferred to a conditioned anode by dipping (89). Excess solvent was evaporated and the anode carrying the sample was introduced into the cool source (ca 80°) through a vacuum lock. When a vacuum of 10^{-6} Torr or better was restored, the high voltage (+3kV to anode and -7kV to cathode) was applied and the focusing elements adjusted using the signal from a field ion beam produced by acetone introduced through the reference inlet. Anode heating was increased until a steady ion beam was obtained on the total ion beam monitor and the magnetic scan was then commenced. Signals were obtained from an electric multiplier (set at 1.75 to 2.0 kV) and the spectra were recorded at a resolution of 1000 (10% Valley definition). The mass scale was calibrated against perfluorokerosene for masses up to 650 and against tris-(perfluoroheptyl)-s-triazine for masses between 650 and 1185 in the e.i. mode. The anode wire was cleaned by gradually increasing the anode current to its maximum value (50 mA).

2) Characterization of Peptides

The peptides were hydrolyzed in 6N HCl at 110° ± 2° for 22 hr. The hydrolyzed peptides were analyzed on the Beckman automatic 120 C amino acid analyzer using the two column procedure. The peptides were also subjected to amino

acid analysis without prior hydrolysis. Thin layer chromatography analyses were carried out on Macherey-Nogel and Co. silica gel plates in three different solvent systems: n-propanol: water (2:1); isoamylalcohol: pyridine: water (35:35:30); butanol: water: acetic acid: ethanol (8:3:1:2). Detection was achieved by spraying the plates with ninhydrin reagent and iodine vapor.

3) Removal of Sodium

(a) Gel Filtration

A column (0.9 x 30 cm) of polyacrylamide gel, Bio Gel P-2 (100 - 200 mesh) was prepared in either 75% ethanol or 0.1 M acetic acid solution. The column was washed with 10 bed volumes of the solution used for preparation of the slurry. Fifty ml of the solvent was passed through the column in order to find if any interfering resin material was eluted. The effluent was freeze dried and the residue was dissolved in 100 µl of distilled deionized water (with < 0.1 ppm sodium as sodium chloride). The resulting solution was analyzed with the field desorption mass spectrometer and no ions were detectable. Then peptide (1 mg) was dissolved in 3 ml of the solvent and applied to the column followed by 1 ml of solvent. Fractions of 2 ml were collected at a flow rate of 0.5 ml min^{-1}. Fractions were analyzed for both peptide and sodium. All tubes including the glass column were silanized.

Method of Test

The fractions were monitored with a Beckman model DU spectrophotometer at either 230 or 280 nm (depending on the

the amino acid composition). The fractions were also monitored with an Unicam Atomic Absorption spectrophotometer model SP 90A operated in the emission mode.

The fractions which contained the peptide were pooled together and lyopilized.

(b) Ion Retardaation

Thirty grams of AG 11A8 ion retardation resin (50 to 100 mesh) self-neutralized form as supplied by Bio Rad Laboratories, were slurried in distilled deionized water (with 0.1 ppm sodium as sodium chloride) and poured into a previously silanized column (0.9 x 42 cm). The column was washed with 5 bed volumes of 1 N ammonium chloride solution. This was followed by deionized water. The washing was continued until no chloride could be detected using 0.1 N silver nitrate solution. Fifty ml of deionized water was then passed through the column in order to find if any interfering resin material was eluted. The effluent was lyophilized and the residue was dissolved in 100 µl of deionized water and analyzed with the field desorption mass spectrometer and no ions were detected. A 10% peptide sample loading on the prepared column was used and 2 ml fractions were collected at a flow rate of 1 ml min^{-1}. Fractions were analyzed for both peptide and sodium. All tubes were silanized.

Method of Test

The fractions were monitored with a Beckman model DU spectrophotometer at either 230nm or 280nm (depending on the

amino acid residue composition of the peptide). The fractions were also monitored with an Unicam SP 90A Atomic Absorption Spectrophotometer operated in the emission mode.

The fractions with the peptide were collected and lyophilized and this was analyzed by the field desorption mass spectrometer.

(c) Ion Exchange

A column (0.9 x 50 cm) of Amberlite IR-120 (hydrogen form) was prepare from 0.1M (in pyridine) pyridine acetate, pH 3.5 $\pm$ 0.02. The column was washed with 5 bed volumes of the buffer. Fifty ml of the buffer was then passed through the column in order to find if any interfering resin material was eluted. The effluent was freeze dried and the residue was dissolved in 100 µl of distilled deionized water or appropriate solvent. The resulting solution was analyzed with the field desorption mass spectrometer and no ions were detectable. Then the peptide (5mg) was dissolved in 5ml of the solvent and applied to the column followed by 1 ml of solvent. Fractions of 2ml were collected at a flow rate of 1ml min^{-1}. All tubs including the column were silanized.

Method of Test

A 0.5ml of effluent with 0.5ml of 0.3% ninhydrin in ethanol was heated in a boiling water bath. The fractions were also monitored with an Unicam SP 90A Atomic Absorption Spectrophotometer operated in the emission mode.

The peptide fractions were pooled together and lyophilized and analyzed with the field desorption mass spectrometer.

CHAPTER III

RESULTS

A. AMINO ACIDS

The field desorption mass spectra, taken at the best emitter current, of the underivatized amino acids studied are presented in Table I. Except in phenylalanine and lysine a quasi-molecular ion $[M+1]^+$ instead of an $[M]^+$ was found as the base peak in the field desorption mass spectra of leucine (mol.wt. 131), tryptophan (mol.wt.204), threonine (mol.wt.119), tyrosine (mol.wt.181) and histidine (mol.wt.155). Peaks due to loss of water (m/e 18) were weak or non-existent. The most intense fragment ion peak was produced by the loss of COOH (m/e 45) and/or $COOH_2$ (m/e 46). A distinction between either a loss of COOH from an unstable $[M]^+$ ion or a loss of $COOH_2$ from a stable $[M+1]^+$ ion was not possible.

1. Threonine, Leucine, Phenylalanine, Tyrosine, Tryptophan and Histidine

The field desorption mass spectra of these amino acids were very similar. The $[M-45]^+$ ion was the only fragment ion detected in all the spectra taken at the best emitter current.

2) Lysine

The field desorption mass spectrum of lysine (Table I) gave a peak at m/e 130 which is due to loss of NH_3 from the $[M+1]^+$ ion. Loss of NH_3 in this spectrum was relatively as important as loss of $COOH_2$ from the $[M+1]^+$ ion. A similar process has been observed for field desorption mass spectrum of arginine (91).

TABLE I Relative Ion Intensities (%) in f.d. mass spectra of Amino Acids

Amino Acids (mol.wt)		$[M-1]^+$ %	$[M]^+$ %	$[M+1]^+$ %	$[M+1]^+ - NH_3$ %	$[M+1]^+ - H_2O$ %	$[M+1]^+ - COOH$ %	$[M+1]^+ - COOH_2$ %
Threonine (119)	a			100.0			17.7	40.0
Leucine (131)	b	8.9	4.5	100.0			42.2	42.2
Lysine (146)	c		100.0	15.3	15.0		10.0	15.5
Histidine (155)	d			100.0			6.8	
Phenylalanine (165)	e		100.0	79.6			45.2	19.9
Tyrosine (181)	f			100.0				5.0
Tryptophan (204)	g			100.0				19.9

a Emitter heating current: 15 mA; Solvent: 75% Ethanol

b Emitter heating current: 12 mA; Solvent: 0.1M Acetic Acid

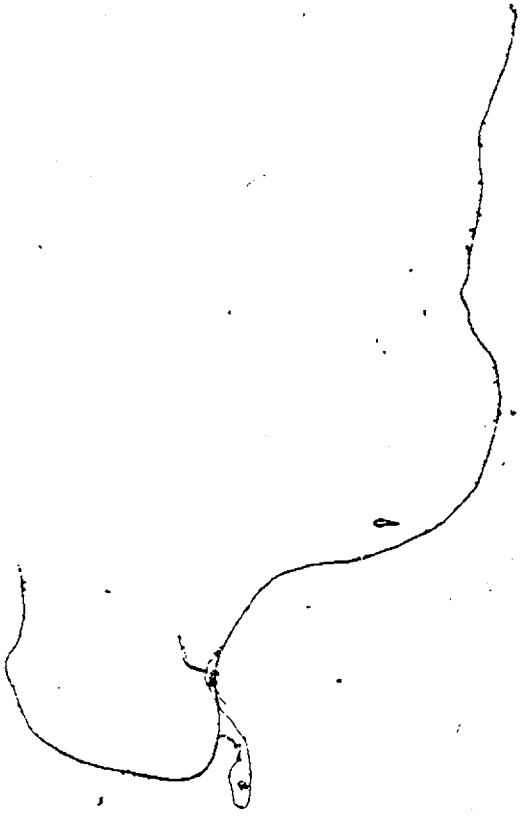
c Emitter heating current: 14 mA; Solvent: 0.1M Hydrochloric Acid

d Emitter heating current: 17 mA; Solvent: 0.1M Hydrochloric Acid

e Emitter heating current: 10 mA; Solvent: Distilled deionized water

f Emitter heating current: 14 mA; Solvent: 75% Ethanol

g Emitter heating current: 15 mA; Solvent: 75% Ethanol



3. Study of the Effect of Emitter Heating Current on f.d.m.s of Amino Acids

The field desorption mass spectra of amino acids do not yield only "two peak spectra" as may be seen in Figure 1. A rapid rise in emitter heating current resulted in a strong fragmentation giving spectra similar to some extent to typical e.i spectra.

The range of emitter heating currents between the beginning of desorption and the complete removal of the amino acids leucine and tryptophan is shown in Tables II and III. The interval of heating current (Δi) passed through the emitter for the desorption of leucine was 4mA (in 25 seconds). The $[M+1]^+$ ion remained the base peak (Table II). The range of currents between the beginning of desorption and the complete removal of tryptophan in 30 seconds is relatively large. The interval of heating currents (Δi) passed through the emitter was 8 mA. The $[M+1]^+$ ion remained the base peak (15 mA to 22 mA) (Table III).

Fig. 1. Field Desorption mass spectrum of phenylalanine.

FIGURE 1

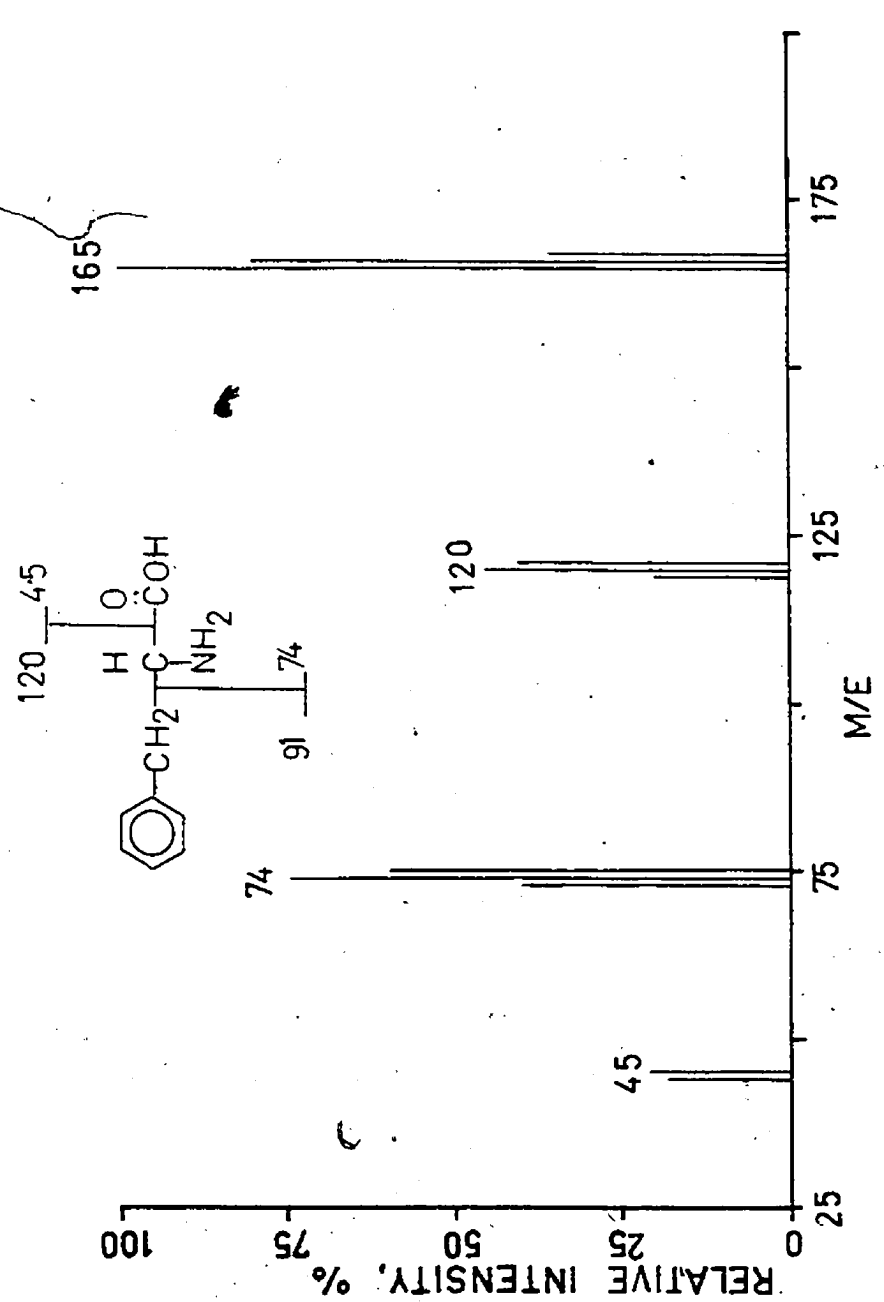


TABLE II: Relative Ion Intensities (%) in f.d. mass spectrum of Leucine (mol.wt. 131) at Different Emitter Heating Currents

m/e	Emitter Heating Currents		
	10mA	12mA	14mA
	Rel.int.(%)	Rel.int.(%)	Rel.int.(%)
86	42.2	34.0	41.5
87	42.2	38.6	27.5
130	11.4	8.9	6.9
131	6.8	4.5	4.3
132	100.0	100.0	100.0
133	13.6	11.1	10.4

A $(2M+1)^+$ ion (rel.int.4.5) was observed at 12mA emitter current.

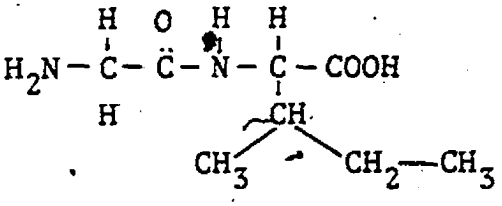
TABLE III: Relative Ion Intensities (%) in f.d. mass spectrum of Tryptophan (mol. wt. 204) at Different Emitter Heating Currents.

m/e	Emitter Heating Currents				
	7mA	10mA	15mA	20mA	22mA
	Rel.int(%)	Rel. int(%)	Rel. int(%)	Rel. int(%)	Rel.int(%).
159	0	0	19.9	0	0
204	0	0	0	0	0
205	0	0	100.0	100.0	100.0
206	0	0	46.5	41.5	41.0

B. DIPEPTIDES

Field desorption mass spectra of the underivatized dipeptides H.Gly.Ile.OH, H.Phe.Val.OH, H.His.Lys.OH, H.His.Ser.OH and H.His.Tyr.OH are given in Tables IV to VIII and fragmentation schemes for the dipeptides are presented in Figures 2 to 6.

1) H.Gly.Ile.OH;



The possible fragmentation scheme showing possible products of unimolecular decomposition in the dipeptide H.Gly.Ile.OH spectrum (Table IV) is presented in Figure 2. A cluster of peaks is centered at m/e 188 $[M]^+$ (mol.wt.188). In f.d. mass spectra peaks in the molecular ion region often occur in clusters due to the addition of a hydrogen atom. In ions with high carbon count the $[M+1]^+$, $[M+2]^+$ and $[M+3]^+$ arise partially from ^{13}C - contributions and partially from hydrogen transfer to the $[M]^+$ and/or $[M+1]^+$ ions. The addition of hydrogen is due to surface and field induced reactions at the anode (91, 122). Less frequently peaks occur with hydrogen atoms abstracted from the ion during the surface reaction (90). As a consequence of these reactions masses cannot be predicted with a certainty of more than plus or minus two mass units.

Peaks occuring at m/e 130 and 159 place isoleucine at the carboxyl terminal while the peak at m/e 30 places glycine

at the amino terminal. The ion of mass m/e 173 is due to loss of CH_3 (m/e 15) from the molecular $[M]^+$ ion. The formation of a diketopiperazine through loss of H_2O (m/e 18) was not detected at the emitter heating current employed (13 mA).

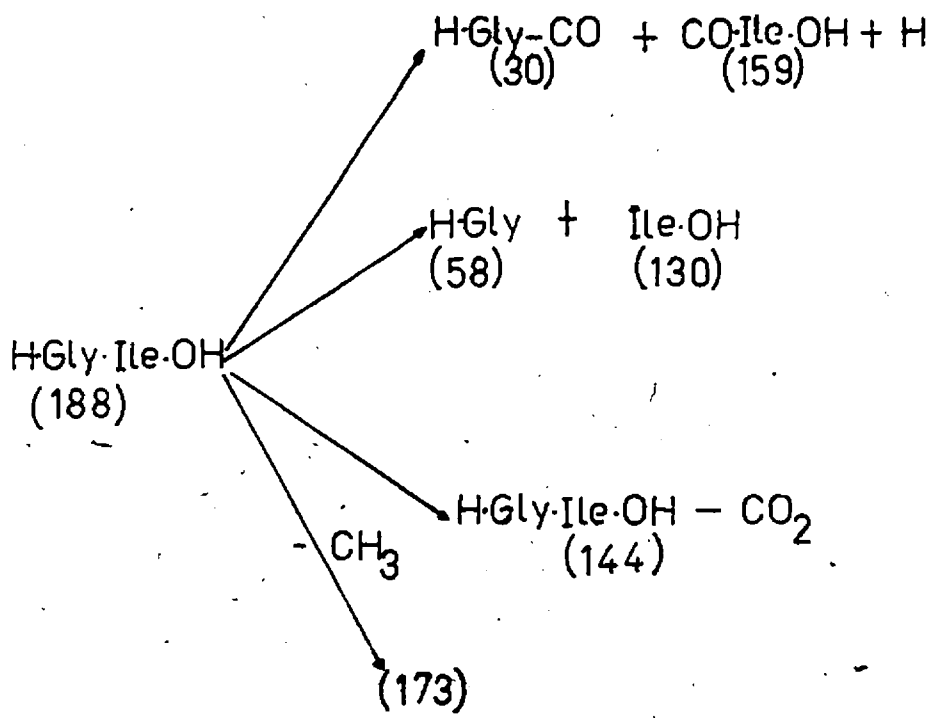
TABLE IV: Relative Ion Intensities (%) in f.d. mass spectrum of H.Gly.Ile.OH (mol.wt. 188.2)

m/e	Rel. int.
29	14.0
30	30.3
129	5.9
130	8.1
143	6.0
144	13.4
145	7.0
158	9.6
159	12.1
173	22.3
174	10.0
188	100.0
189	34.2
190	12.0

Emitter Heater Current - 13 mA. Solvent: Ethanol: Water (75:25) All ions of relative abundance greater than 5% are reported.

Fig. 2: Fragmentation scheme for underivatized dipeptide, H.Gly.Ile.OH, showing possible products of decomposition.

FIGURE 2



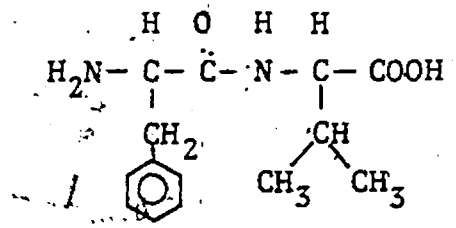
2) <u>H.Phe.Val.OH</u>; 

Table V contains the mass spectrum of the dipeptide H.Phe.Val.OH (mol.wt. 264.3) and Figure 3 the corresponding fragmentation pattern. A cluster of peaks centered at m/e 265($[M+1]^+$) appeared as the base peak. The peak at m/e 146 (144 +2H) places valine at the carboxyl terminal while the peaks at m/e 120 and 148 place phenylalanine at the amino terminal. Loss of $COOH_2$ from $[M+1]^+$ was observed at m/e 219. The intense peaks at m/e 92 and 172 correspond to benzyl plus a hydrogen atom $[C_6H_5CH_2 + H]^+$ and loss of benzyl group from the $[M+1]^+$ ion. Cleavage between the valine side chain and the peptide backbone produced the intense peaks at m/e 43 ($CH_3-CH-CH_3$) and m/e 221. The ion at m/e 249 is due to loss of CH_3 (m/e 15) from a $[M]^+$ ion. The formation of a diketopiperazine was not observed at the emitter heating current used in this study (14 mA).

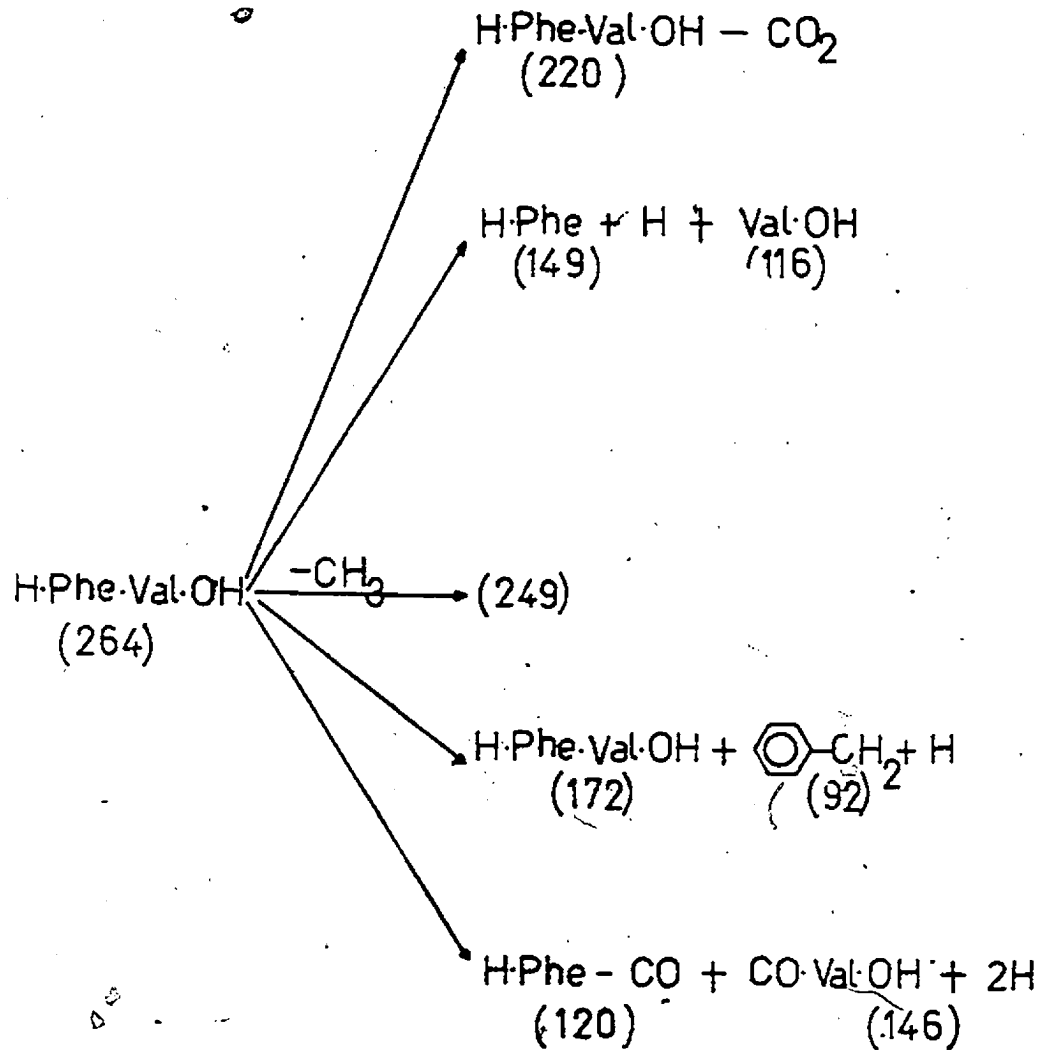
TABLE V: Relative Ion Intensities (%) in f.d. mass spectrum of H.Phe.Val.OH (mol.wt. 264.3)

m/e	Rel. int.
43	33.0
44	11.8
45	15.0
91	17.9
92	20.0
120	18.0
121	9.9
133	12.0
146	10.6
148	16.0
149	10.0
172	22.5
173	12.1
219	11.9
220	15.5
221	10
248	18.8
249	22.4
250	9.1
264	60.0
265	100.0
266	36.0

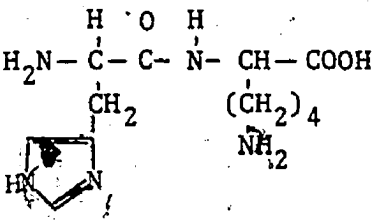
Emitter heating current ~14 mA. Solvent: Ethanol: Water (75:25). All ions of relative abundance greater than 5% are reported.

Fig. 3: Fragmentation scheme for underivatized dipeptide, H.Phe.Val.OH, showing possible products of decomposition.

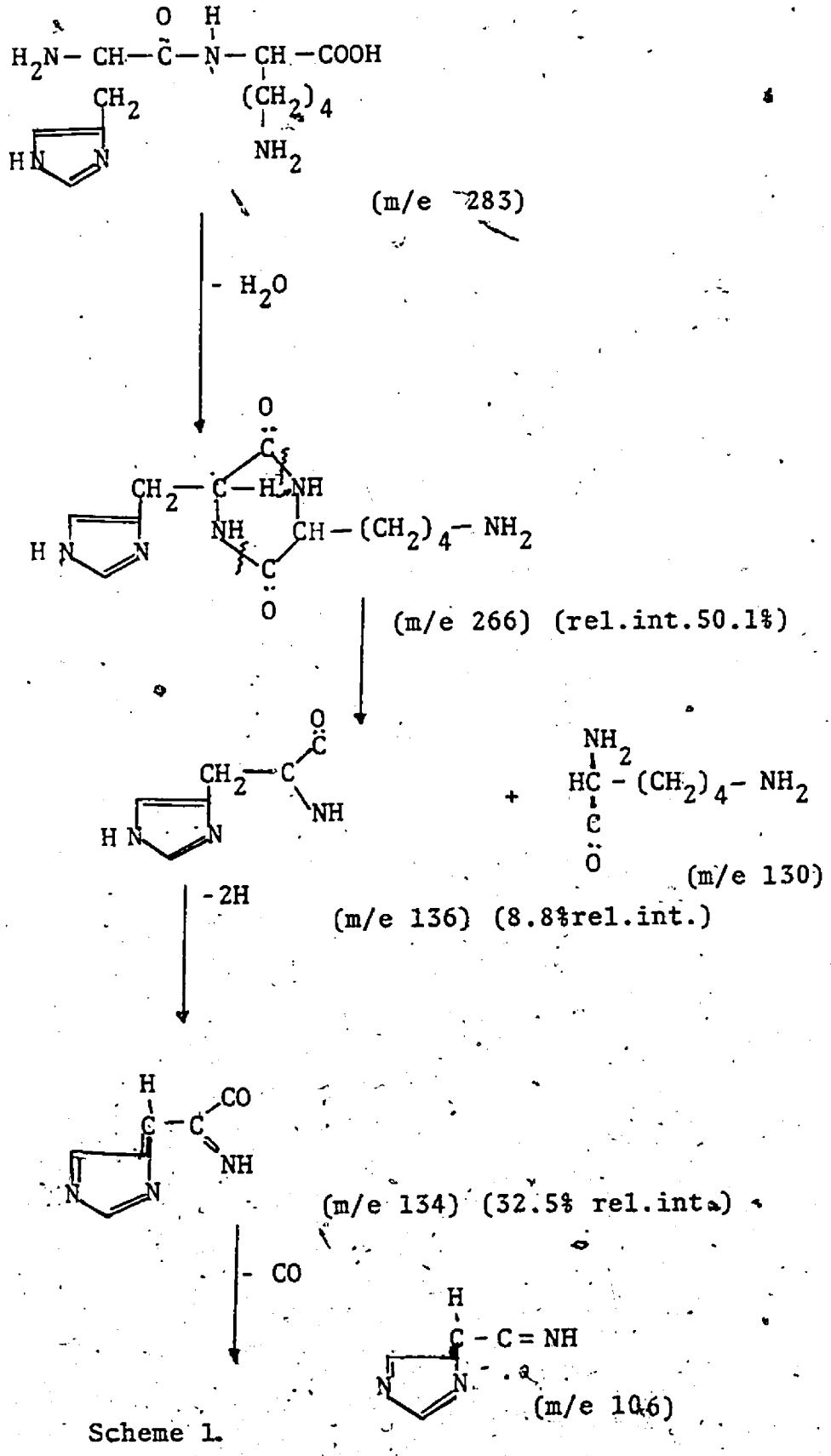
FIGURE 3



3) H.His.Lys.OH;



A molecular ion cluster at m/e 284 ($[M+1]^+$) was observed for the dipeptide H.His.Lys.OH (mol.wt. 283.4) (Table VI). Figure 4 presents the fragmentation scheme. The base peak at m/e 143 is assigned to the fragment $(Lys.OH-2H)^+$. The relatively intense peak at m/e 134 (32% rel.int.) cannot be rationalized on the basis of a simple fragmentation process. The peaks at m/e 18 (8.8% rel.int.) and 266 (50% rel.int.) ($[M+1]^+ - H_2O$) are sufficient evidence to show that a diketopiperazine has been formed. A similar process has been observed for H.Gly.Gly.OH (100). The peak at m/e 134 may be formed according to Scheme 1.



Scheme 1.

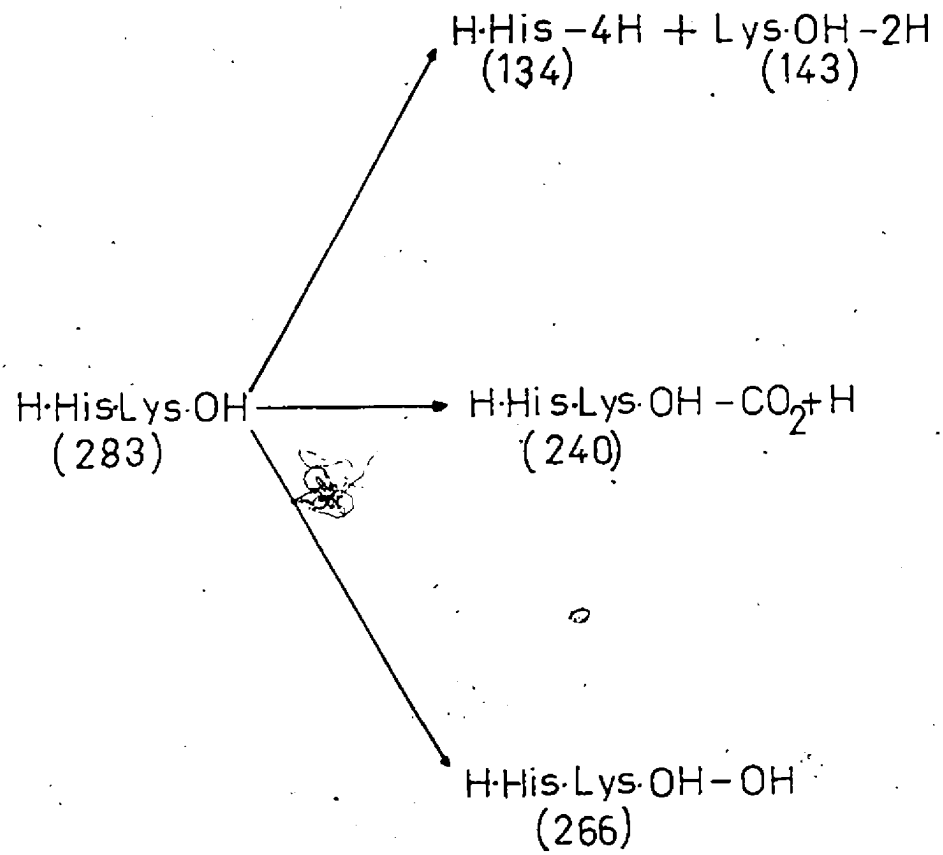
TABLE VI: Relative Ion Intensities (%) in f.d. mass spectrum of H.His.Lys.OH (mol.wt. 283.4).

m/e	Rel. int.
134	32.5
135	30.0
136	8.8
143	100.0
144	31.2
149	8.8
150	10.0
240	7.5
266	50.1
267	10.0
270	8.8
284	70.0
285	15.0

Emitter Heating Current ~ 20 mA. Solvent: Ethanol: Water (75:25). At higher emitter heating current m/e 266 became the base peaks. All ions of relative abundance greater than 5% are reported.

Fig. 4. Fragmentation scheme for underivatized dipeptide, H.His.Lys.OH, showing possible products of decomposition.

FIGURE 4



4) <u>H.His.Ser.OH</u>;

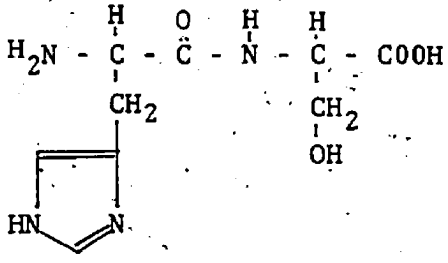


Table VII presents the mass spectrum of the dipeptide H.His.Ser.OH (mol.wt. 242.2) and Figure 5 rationalizes the fragmentation pattern. The significant features of the spectrum are the cluster of ions of m/e 243 corresponding to the $[M+1]^+$ ion and the intense peak at m/e 225 which is due to loss of H_2O from the $[M+1]^+$ ion and has the mass of a protonated dikeptopiperazine. Loss of $COOH_2$ from $[M+1]^+$ gave the ion at m/e 197 while loss CO_2 produced the ion at m/e 199. The ion at m/e 133 (132 + H) places serine (CO.Ser.OH + H) at the carboxyl terminal. The cluster of ions at m/e 156 corresponds to the free histidine (impurity). From the thin layer chromatography of the peptide no histidine peak could be detected. Perhaps histidine arises from hydrolysis of the peptide on the wire.

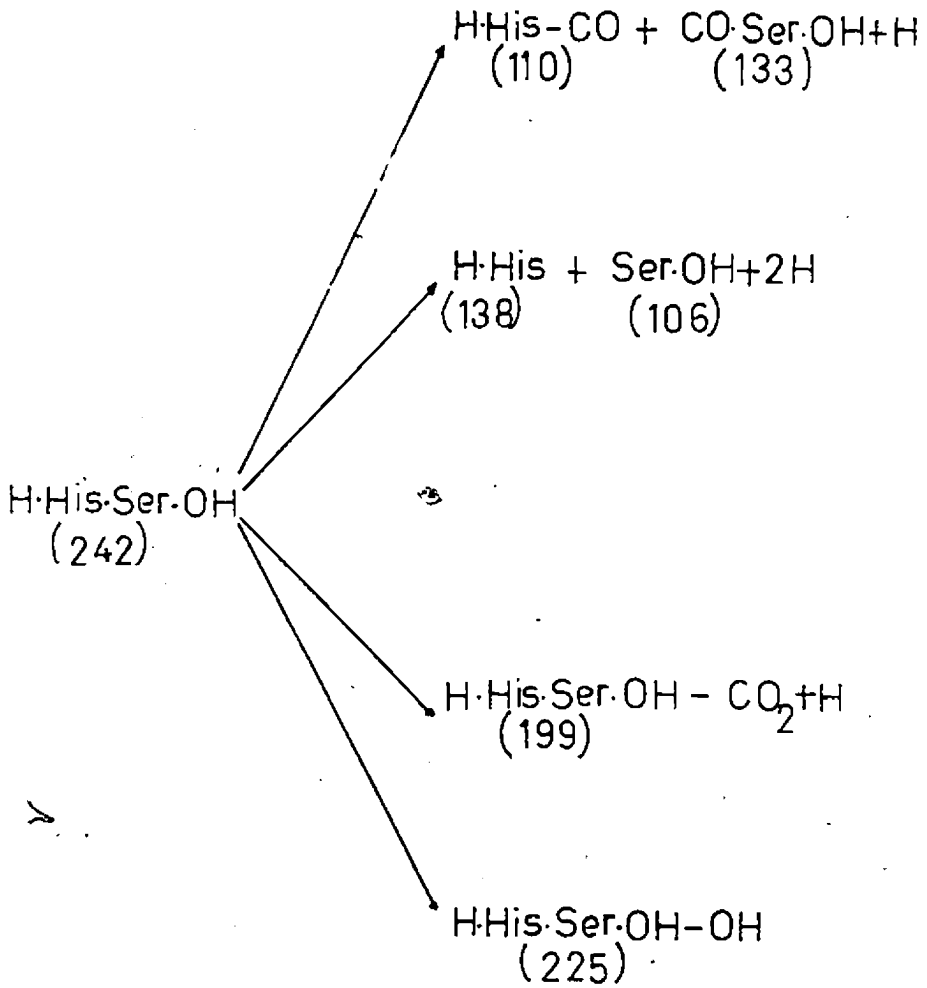
TABLE VII: Relative Ion Intensities (%) in f.d. mass spectrum of H.His.Ser.OH (mol.wt. 242.2)

m/e	Rel. int.
90	2.4
106	3.9
133	1.8
155	2.7
156	11.3
157	1.8
197	1.8
199	2.4
207	2.9
225	39.5
226	13.1
243	100.0
244	13.1
245	3.6

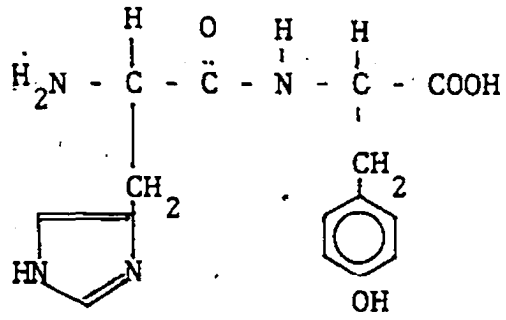
Emitter heating current ~18.6 mA. Solvent: 0.1 M Acetic acid. All ions of relative abundance greater than 1.5% are registered.

Fig. 5. Fragmentation scheme for underivatized dipeptide, H.His.Ser.OH, showing possible products of decomposition.

FIGURE 5



5) H.His.Tyr.OH;



The highest molecular weight species observed in the H.His.Tyr.OH (mol.wt. 318.1) spectrum was m/e 301 (Table VIII). It is formed by loss of H_2O from the protonated parent molecular ion m/e 319 (4.7% rel.int.). It is reasonable to assume that the base peak at m/e 134 did not form from the dipeptide by simple rupture of the peptide bond with hydrogen rearrangement. It could be formed according to Scheme 1.

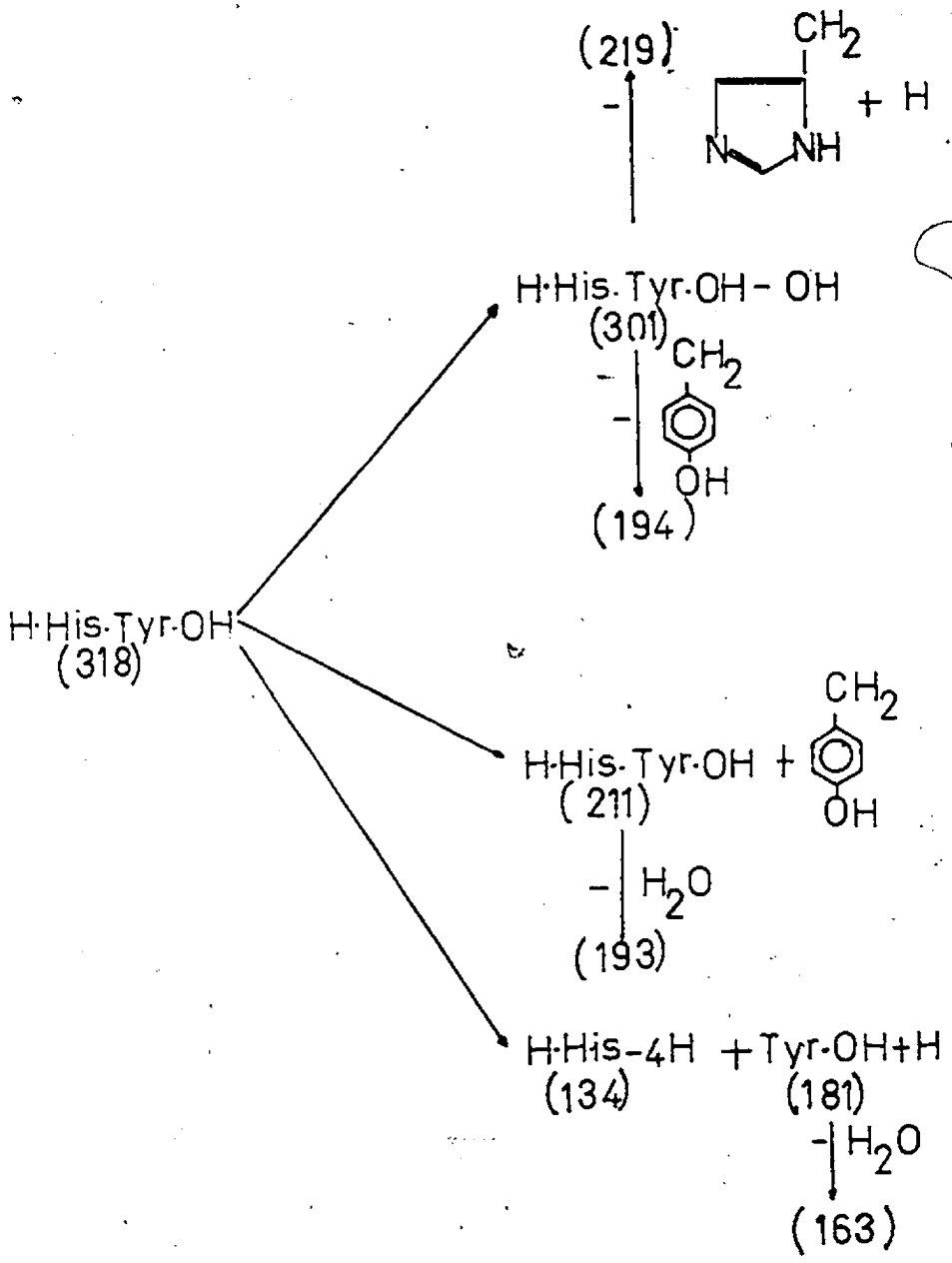
TABLE VIII: Relative Ion Intensities (%) in f.d. mass spectrum of H.His.Tyr.OH (mol.wt. 318.1).

m/e	Rel. int.
77	7.0
88	7.2
105	9.3
106	77.0
107	7.1
134	100.0
150	9.3
151	7.4
300	11.6
301	21.0
302	7.0
319	4.7

Emitter heating current 18 mA. Solvent: Ethanol:Water (75:25). Other peaks of interest were m/e 163 (4.8%), 193 (2.3%), 194 (4.7%), 219 (4.6%) and 156 (2.5%). All ions of relative abundance greater than 5% are registered.

Fig. 6. Fragmentation scheme for underivatized dipeptide, H.His.Tyr.OH, showing possible products of decomposition.

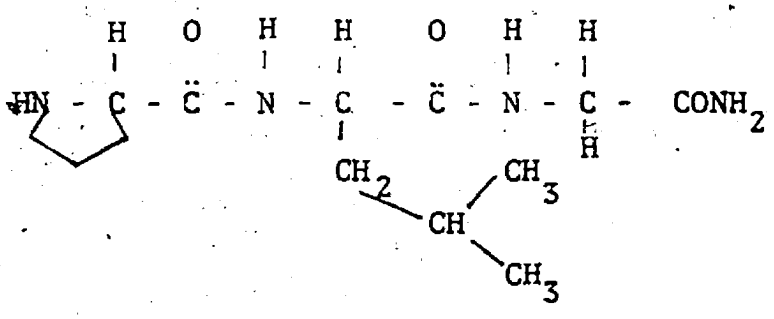
FIGURE 6



C. TRIPEPTIDES:

The mass spectra of three tripeptides, H.Pro.Leu.Gly.NH_2 (mol.wt.284.2), H.(HO-γ-Glu).Cys.Gly.OH (mol.wt.307.2) and H.Met.Ala.Ser.OH (mol.wt.307.4) are given in Table IX to XI and Figures 7 to 9 contain the corresponding fragmentation patterns. The spectra conform to a general pattern. There is at the high-end of the spectrum a cluster of peaks centered at an $[M]^+$ or $[M+1]^+$ ion which in each case was the base peak. The peptide chains were cleaved preferentially at the peptide bonds. Intense peaks corresponding to the amino terminal and the carboxyl terminal amino acid residues with or without hydrogen atoms transferred to the ion were observed. The sequence of a tripeptide is established by a relatively strong set of ions corresponding to an amino terminal amino acid and carboxyl terminal dipeptide fragment as well as a carboxyl terminal amino acid and an amino terminal dipeptide fragment. The other ions of significance (Table IX to XI) can be accounted for by decomposition processes involving loss of small stable molecules from the quasimolecular ions $[M+1]^+$ such as H_2O, NH_3, $COOH_2$, CH_3OH, CH_3SH, $CONH_3$ and CO_2.

1) H.Pro.Leu.Gly.NH_2:



A cluster of peaks is centered at m/e 285 ($[M+1]^+$) of the tripeptide H.Pro.Leu.Gly.NH_2 (mol.wt.284.2) (Table IX). Peaks occurring at m/e 216 (214 + 2H), 186, 101, 74 (73 + H) and 70 in the spectrum can be related to the sequence of the tripeptide (Figure 7). The ion of mass 242 is due to the loss of - $CONH_2$ from the $[M+2]^+$ ion. It could also arise by loss of part of luecine side chain as CH_3 - CH -CH_3 (m/e 43) from $(M+1)^+$ ion.

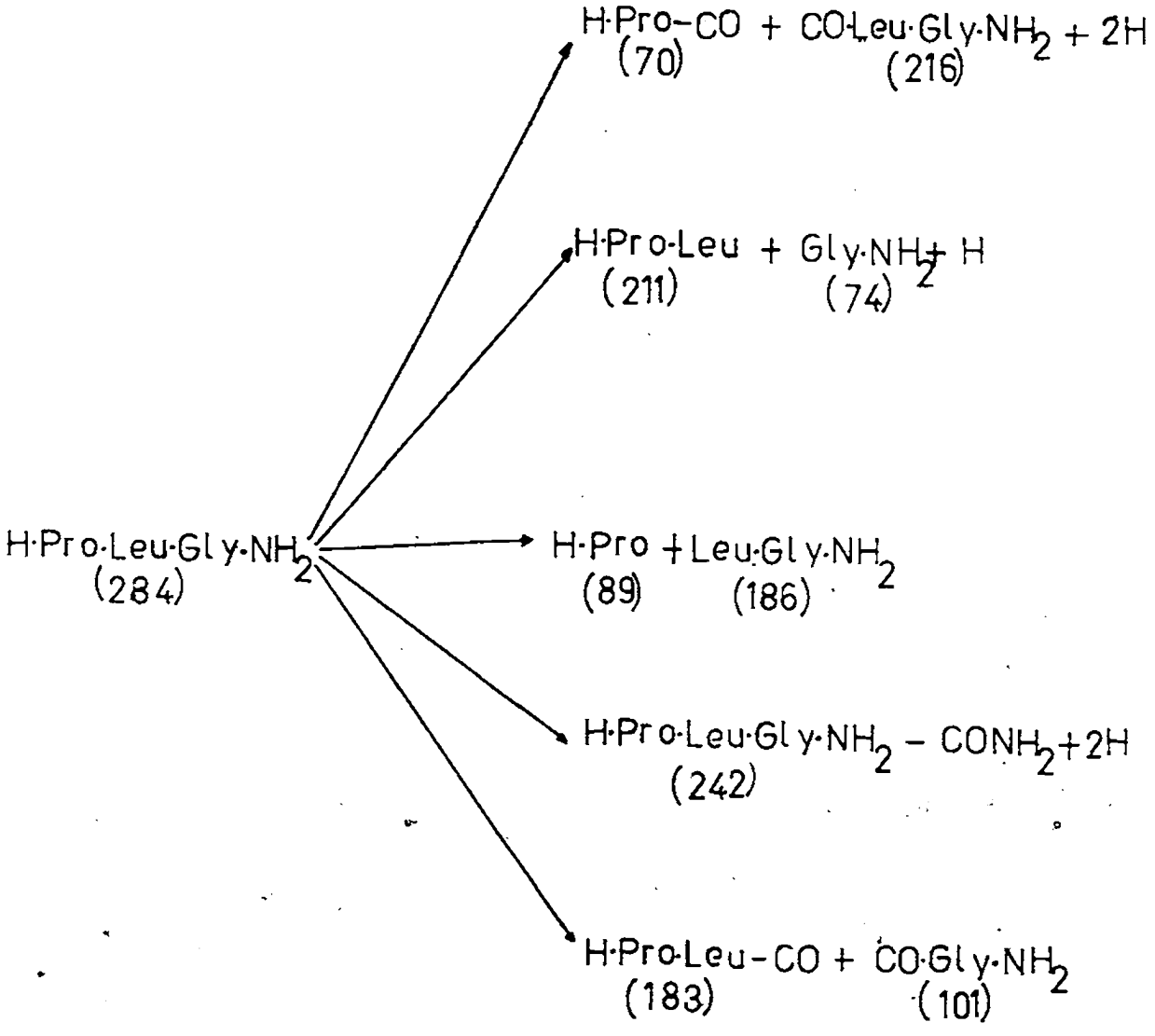
Table IX: Relative Ion Intensities (%) in f.d. mass spectrum of H.Pro.Leu.Gly.NH_2 (mol.wt.284)

m/e	Rel.int.
70	20.0
74	19.6
101	19.4
186	19.9
216	11.2
242	5.6
260	22.4
284	16.8
285	100.0
286	93.0
287	48.6

Emitter heating current ~ 10 mA. Solvent: Ethanol: Water (75:25). All ions of relative abundance greater than 5% are registered.

Fig. 7. Fragmentation scheme for underivatized tripetide, H.Pro.Leu.Gly.NH_2, showing possible products of decomposition.

FIGURE 17



2) H.(HO - γ - Glu). Cys. Gly. OH:

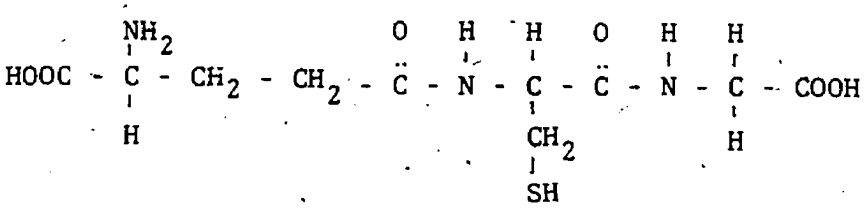


Table X shows the mass spectrum of the tripeptide containing a γ-glutamic acid residue: H (HO - γ - Glu). Cys.Gly.OH (mol.wt.307.2). The $(M+1)^+$ ion was the base peak. Amino terminal sequence ions occur at m/e 130 (H(HO- γ - Glu), 204(H(HO - γ - Glu).Cys – CO–H) and 233 (H.HO -γ -Glu).Cys), while carboxyl terminal sequence ions occur at m/e 76 (Gly.OH + 2H),102 (CO.Gly.OH) and 177 (Cys.Gly.OH). Figure 8 rationalizes the production of the ions. The fragment ion at m/e 290 is accounted for by loss of H_2O from the $[M+1]^+$ ion. Other secondary processes such as loss of SH, CO_2, CH_3SH, and $COOH_2$ occur as significant processes draining the sequence ions. Isobaric fragment ions occurred at m/e 102 and 204. The ion at m/e 102 is assigned to the fragment CO.Gly.OH (m/e 102) as proposed for the case of electron impact by Van Heijenoort et al (123) rather than the fragment HOOC-CH(NH_2) -CH_2-CH_2 (m/e 102). The ion at m/e 204 could be related to the ion H.(HO -γ - Glu).Cys – CO – H or CO.Cys.Gly.OH-H.

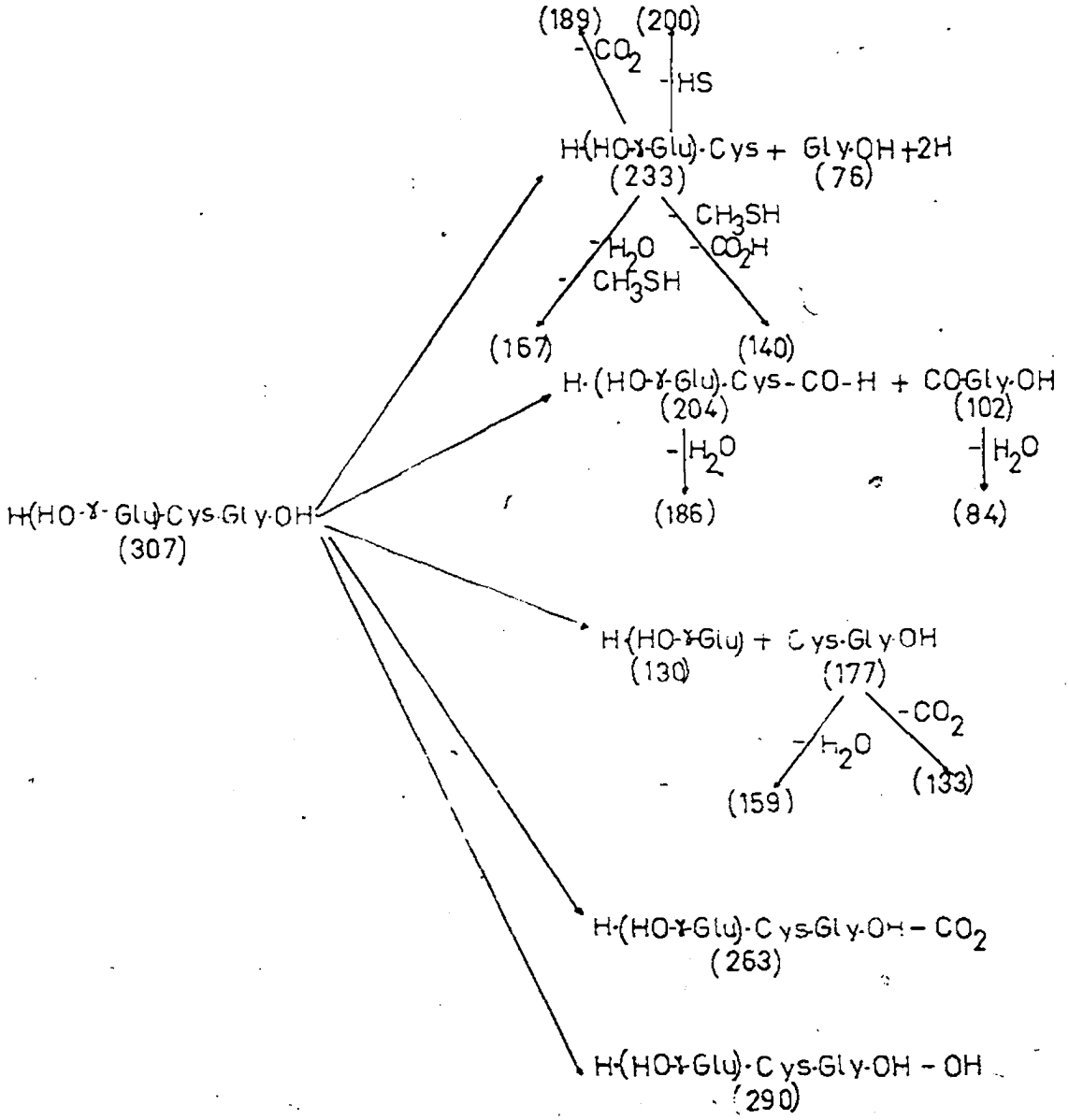
Table X: Relative Ion Intensities (%) in f.d. mass spectrum of H. (HO -γ - Glu).Cys.Gly.OH (Mol.wt.307.2)

m/e	Rel.int.	m/e	Rel.int.
71	14.2	186	14.3
76	12.0	189	14.2
84	19.0		
98	9.5	200	9.5
102	9.5	203	12.0
115	12.1	204	14.2
116	9.5		
117	14.3	217	9.5
129	14.2	220	7.2
130	33.4		
131	12.0	233	7.3
133	16.7	243	12.0
136	14.2	244	7.2
140	14.2	245	7.2
141	12.1		
		263	16.7
145	9.5	264	14.2
146	9.6	265	12.0
159	14.2	286	12.0
167	9.5	290	21.5
		291	16.7
176	24.0	292	12.0
177	45.4		
178	31.0	300	12.0
179	16.7		
		307	16.7
		308	100.0
		309	28.6
		310	16.7

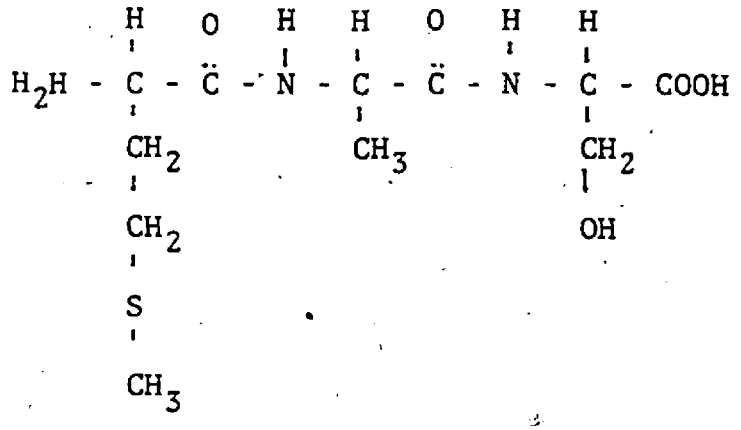
Emitter heating current 20 mA. Solvent: Deionized distilled water (with < 0.1 ppm. sodium as sodium chloride). All ions of relative abundance greater than 5% are registered.

Fig. 8. Fragmentation scheme for underivatized tripeptide, H.(HO-γ-Glu).Cys.Gly.OH, showing possible products of decomposition.

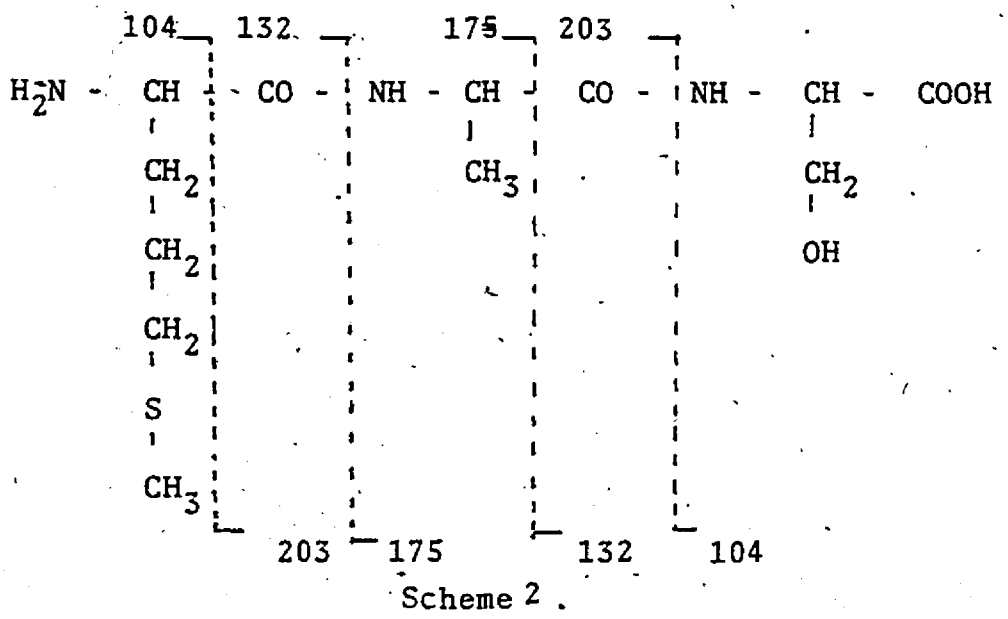
FIGURE 8



3). H.Met.Ala.Ser.OH:



A cluster of peaks centered at m/e 307 ($[M]^+$) was observed in the spectrum of the tripeptide, H.Met.Ala.Ser.OH (mol.wt.307.4) (Table XI). Sequence peaks due to cleavage in the backbone of the peptide occur at m/e 103, 104,106(104+2H), 132,203 and 204 (Figure 9). The tripeptide H.Met.Ala.Ser.OH illustrates a problem arising from isobaric masses of combinations of amino acids. Scheme 2 illustrates the problem.



Scheme 2.

Studies of isotopic peaks of sulfur in the ions 103 and 105, 132 and 134, 203 and 205 were essential for the correct assignment of the isobaric ions. The abundance of ions at m/e 105 is about 3.8% of m/e 103 which can account for the natural abundance of ^{34}S (4%). The abundance of ions at m/e 134 is less than 1% of m/e 132 and much too low to account for the natural abundance of ^{34}S. The isotopic peak at m/e 205 corresponds to about 4% of the peak at m/e 203 indicating one sulfur atom in the ion or a H.Met.Ala structure. The peak at m/e 105 could also arise from addittion or abstraction of a hydrogen atom from m/e 104 or m/e 106 respectively. Similarly, the peak at 205 could arise from addition of a hydrogen atom to the ion at m/e 204. The peaks at m/e 104, 106 (104 + 2H) and 132 place serine at the carboxyl terminal of the tripeptide. The amino terminal amino acid was identified as methionine by the peak at m/e 103. The peak at m/e 204 places the dipeptide (H.Met.Ala + H) at the amino terminal. Loss of CH_3OH (m/e 32) from the $[M]^+$ ion produced the peak at m/e 275. Loss of a methyl mercaptan (m/e 48) from $[M+1]^+$ accounts for the ion at m/e 260. The mass ion at m/e 290 is due to the loss of H_2O from $[M+1]^+$. With the exception of ions at m/e 270 and 144 the decomposition scheme in Figure 9 provides a basis for the identification of the bulk of the mass spectrum.

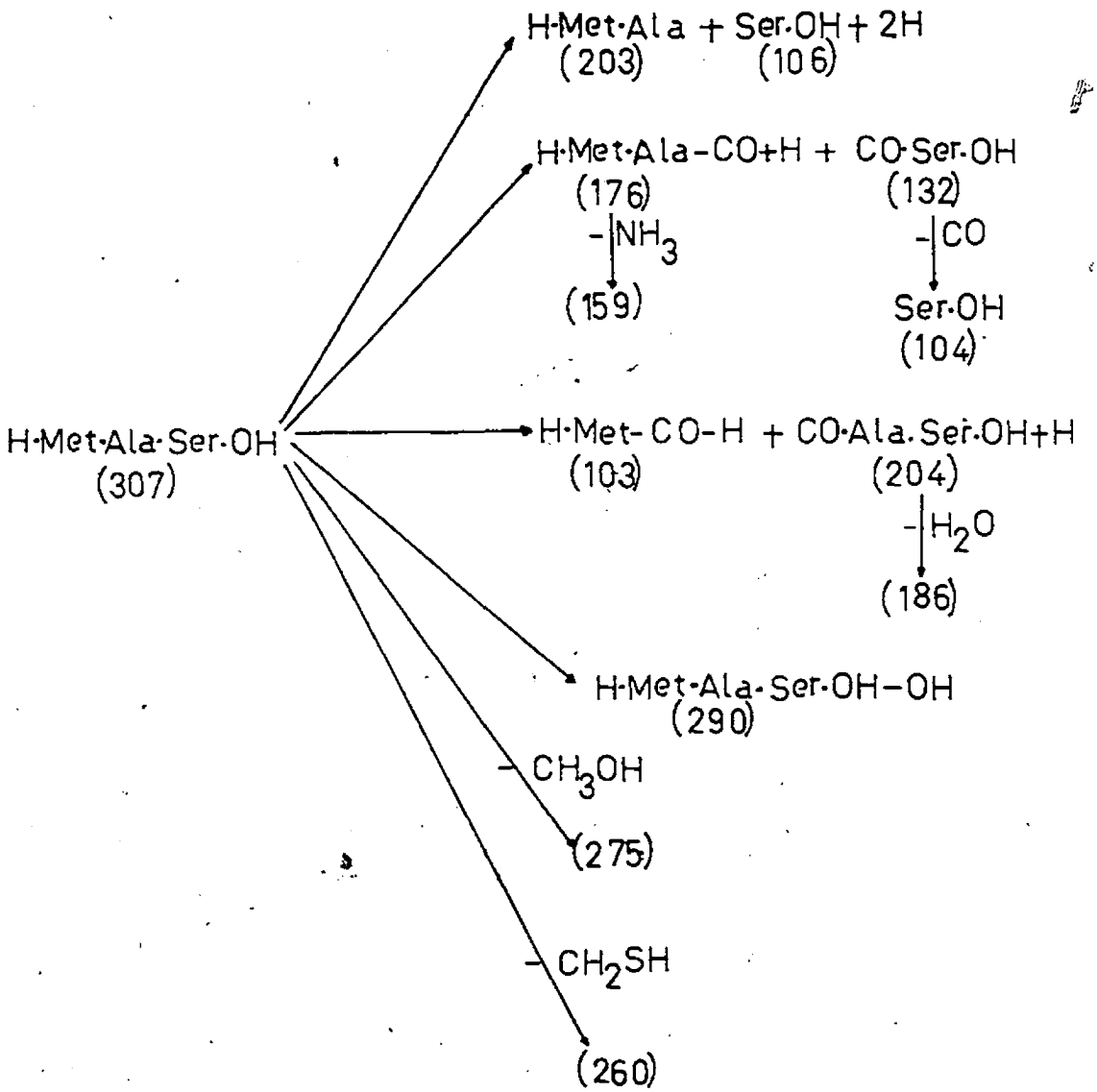
Table XI: Relative Ion Intensities (%) in f.d. mass spectrum H.Met.Ala.Ser.OH (mol.wt. 307).

m/e	Rel.int.
103	12.0
104	24.2
106	24.0
132	12.2
144	16.4
159	12.0
186	20.1
203	20.3
204	12.1
260	20.0
270	12.3
275	16.0
290	8.4
307	100.0
308	48.1
309	20.3
310	16.3

Emitter heating current ~ 23 mA. Solvent: 0.1M Acetic acid. All ions of relative abundance greater than 5% are registered.

Fig. 9. Fragmentation scheme for underivatized tripeptide, H.Met.Ala.Ser.OH, showing possible products of decomposition.

FIGURE 9



D. TETRAPEPTIDES; PENTAPEPTIDES, HEXAPEPTIDES.

Identification of fragments in the field desorption spectra of large peptides is no more difficult than that encountered with the dipeptides and the tripeptides. Again the addition and loss of hydrogens by the peptides in surface reactions produce a cluster of ions in the molecular ion region.

1) H.Trp.Met.Asp.Phe.NH_2

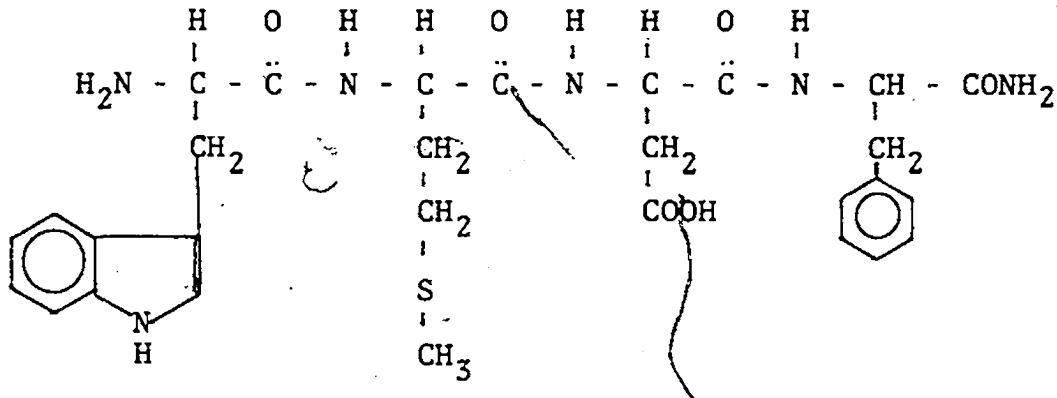
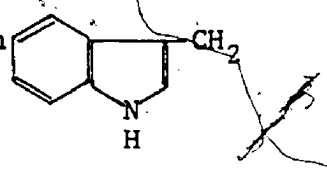


Table XII shows the field desorption spectrum of the tetrapeptide H.Trp.Met.Asp.Phe.NH_2 (mol.wt. 596.2). The base peak was observed at m/e 597 ($[M+1]^+$). The spectrum establishes Phe.NH_2 as the carboxyl terminal amino acid residue by the peak at m/e 164. This assignment is supported by the failure to observe the alternative carboxyl terminal aspartic acid (m/e 132), methionine (m/e 148) or tryptophan (m/e 203) residue. The peak at m/e 119 is due to loss of the elements of $HCONH_2$ (m/e 45) from the carboxyl-terminal Phe.NH_2. The peak at m/e 188 places tryptophan at the amino terminal.

A peak at m/e 130 is due to the methylindol fragment of tryptophan



The peaks at m/e 318 and 278 correspond to the dipeptide fragment H.Trp.Met and Asp.Phe.NH_2 respectively, while the peaks at m/e 434 and 437 indicate the tripeptide fragments H.Trp.Met.Asp and CO.Met.Asp.Phe.NH_2 respectively. The overlap of the tripeptide fragments (H.Trp.Met.Asp. and CO.Met.Asp.Phe.NH_2) establishes the sequence of the tetrapeptide as H.Trp.Met.Asp.Phe.NH_2. Corroboration is provided by the dipeptide fragments at m/e 318 (H.Trp.Met) and 278 (Asp.Phe.NH_2). This conclusion is supported by the failure to observe ions corresponding to the dipeptide fragments H.Trp.Asp (m/e 302)and/or H.Trp.Asp - CO (m/e 274) or H.Trp.Phe (m/e 334) and/or H.Trp.Phe - CO (m/e 306). The abundance of all sequence ions is below 20% relative to the base peak. This may be due to secondary processes including loss of H_2O, HCOOH, $CONH_3$, and CH_3SH which drain ion current from sequence determining ions (Figure 10).

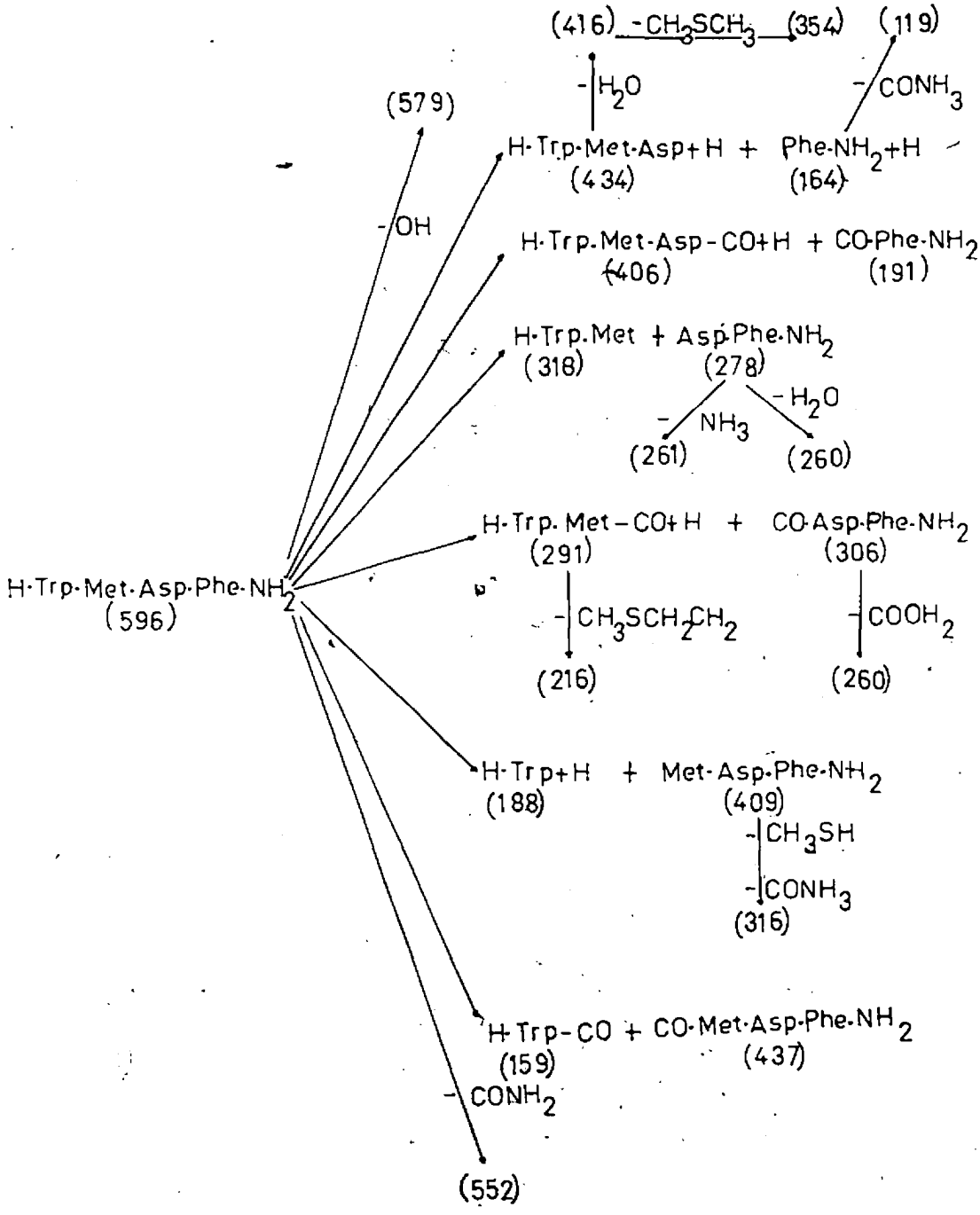
Table XII: Relative Ion Intensities (%) in f.d. mass spectrum of H.Trp.Met.Asp.Phe.NH_2 (mol.wt.596).

m/e	Rel.int.	m/e	Rel.int.
87	8.2	465	5.4
119	5.5	473	5.5
130	6.9	482	8.2
164	5.5	490	5.5
188	4.1	522	8.2
216	4.1	532	6.9
260	5.5	535	4.1
261	5.5	536	5.5
278	6.9	550	6.9
279	5.4	551	5.6
314	5.5	552	8.3
316	12.4	553	9.6
318	8.2	554	9.6
319	8.2	555	19.2
354	5.4	561	8.2
406	4.1	562	8.2
414	4.2	579	61.8
416	4.3	580	15.0
433	4.1	597	100.0
434	5.6	598	49.3
437	3.0	599	15.0
450	4.3		
451	5.6		
452	5.5		

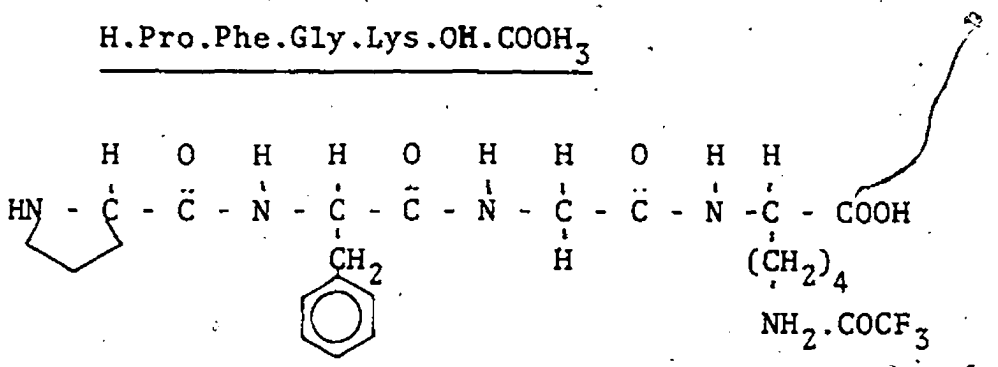
Emitter heating current 19mA. Solvent: Ethanol:Water (75:25). All ions of relative abundance greater than 3% are registered.

Fig. 10. Fragmentation scheme for underivatized tetrapeptide, H.Trp.Met.Asp.Phe.NH_2, showing possible products of decomposition.

FIGURE 10



2. H.Pro.Phe.Gly.Lys.OH.$COOH_3$



This peptide was supplied to us as the acetate (mol.wt.507.3). The field desorption mass spectrum (Figures 11 a,b,c,) showed no peak at m/e 507 but rather a peak at m/e 543 corresponding to the triflouroacetyl derivative of the tetrapeptide. The base peak was observed at m/e 447 which corresponds to the protonated peptide (without the trifluoroacetyl group, that is, Peptide.$COCF_3$-$COCF_3$+H). The sequence is defined by the series of peaks occurring at m/e 70 (H.Pro-CO), 216(217-H; H.Pro.Phe-CO-H), 245 (H.Pro.Phe),276 (274+2H;H.Pro.Phe.Gly-CO+2H), and 302 (H.Pro.Phe.Gly.), corresponding to the amino terminal sequence fragments. Fragments from the carboxyl terminus are found at m/e 377(376+H;CO.Phe.Gly.Lys.OH+H), 350(348+2H; Phe.Gly.Lys.OH+2H), 229(CO.Gly.Lys.OH), 203(201+2H; Gly.Lys.OH+2H), 175(173+2H; CO.Lys.OH+2H). All the carboxyl terminal sequence ions are accompanied by satellite peaks 97 mass units higher due to retention of the trifluoroacetyl group from the protected lysine residue. The peak at m/e 241 is assigned to the fragment Lys.OH.$COCF_3$ and that at m/e 270 assigned to CO.Lys.OH.$COCF_3$+H. The peaks at m/e 298 and 327 are assigned

to the fragments Gly.Lys.OH.$COCF_3$ and CO:Gly.Lys.OH.$COCOF_3$, respectively. Similarly the peak at m/e 445 is assigned to the fragment Phe.Gly.Lys.OH.$COCOF_3$ and at m/e 473 assigned to CO.Phe.Gly.Lys.OH.$COCOF_3$. At the relatively higher emitter heating current (22 mA) employed for the desorption of the peptide, more thermal energy was transferred to the peptide resulting in a strong fragmentation and giving a spectrum similar to some extent to typical electron impact. The dimer $(2M)^+$ (rel.int.4.9) and a protonated dimer $(2M + 1)^+$ (rel.int.4.1) were observed at m/e 894 and 895 respectively (Figure 11c). The possible fragmentation pattern is shown in Figure 12.

Fig. 11a : Field Desorption mass spectrum of H.Pro.Phe.Gly.Lys.OH. $COCF_3$ at 16mA.

FIGURE 11a

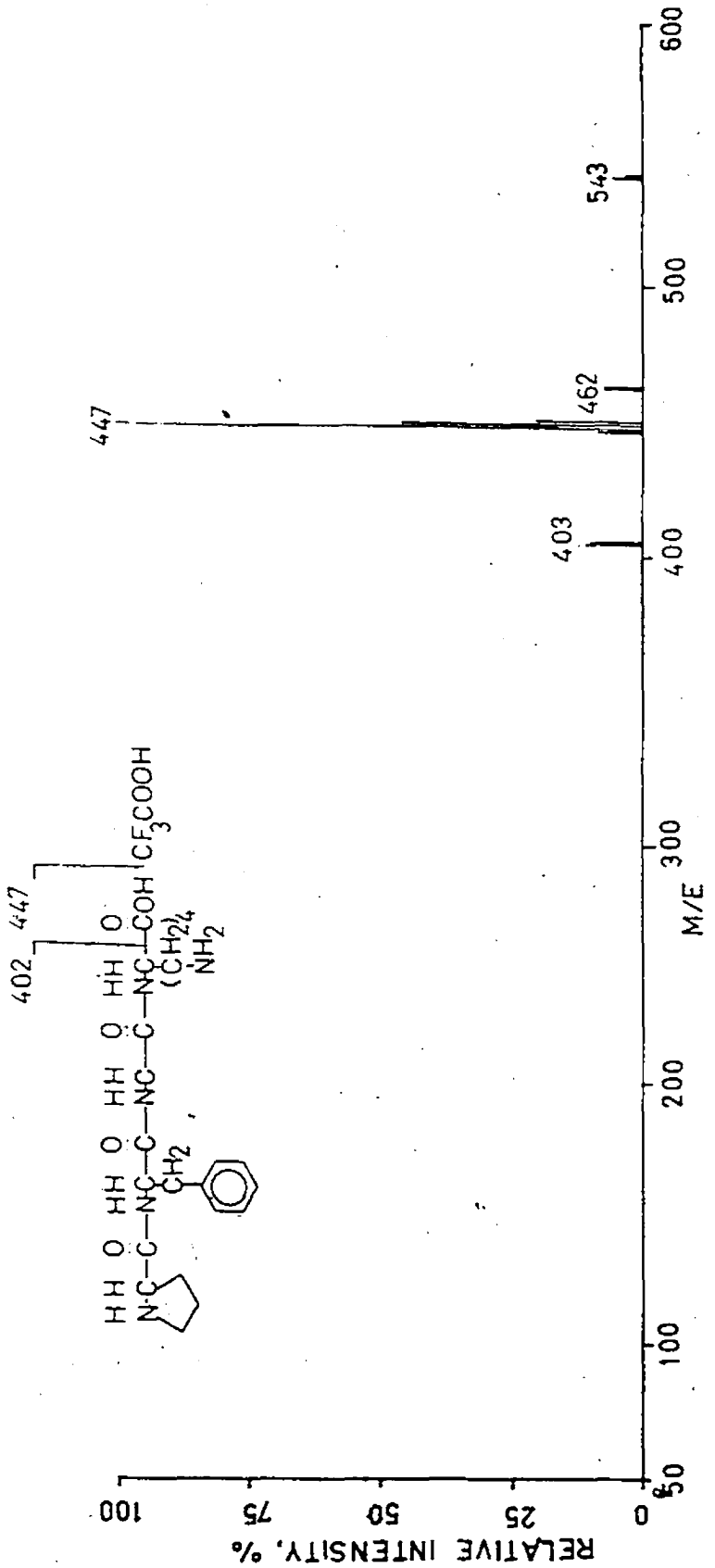


Fig. 11b : Field Desorption mass spectrum of H.Pro.Phe.Gly.Lys.OH. $COCF_3$ at 18mA.

FIGURE 11b

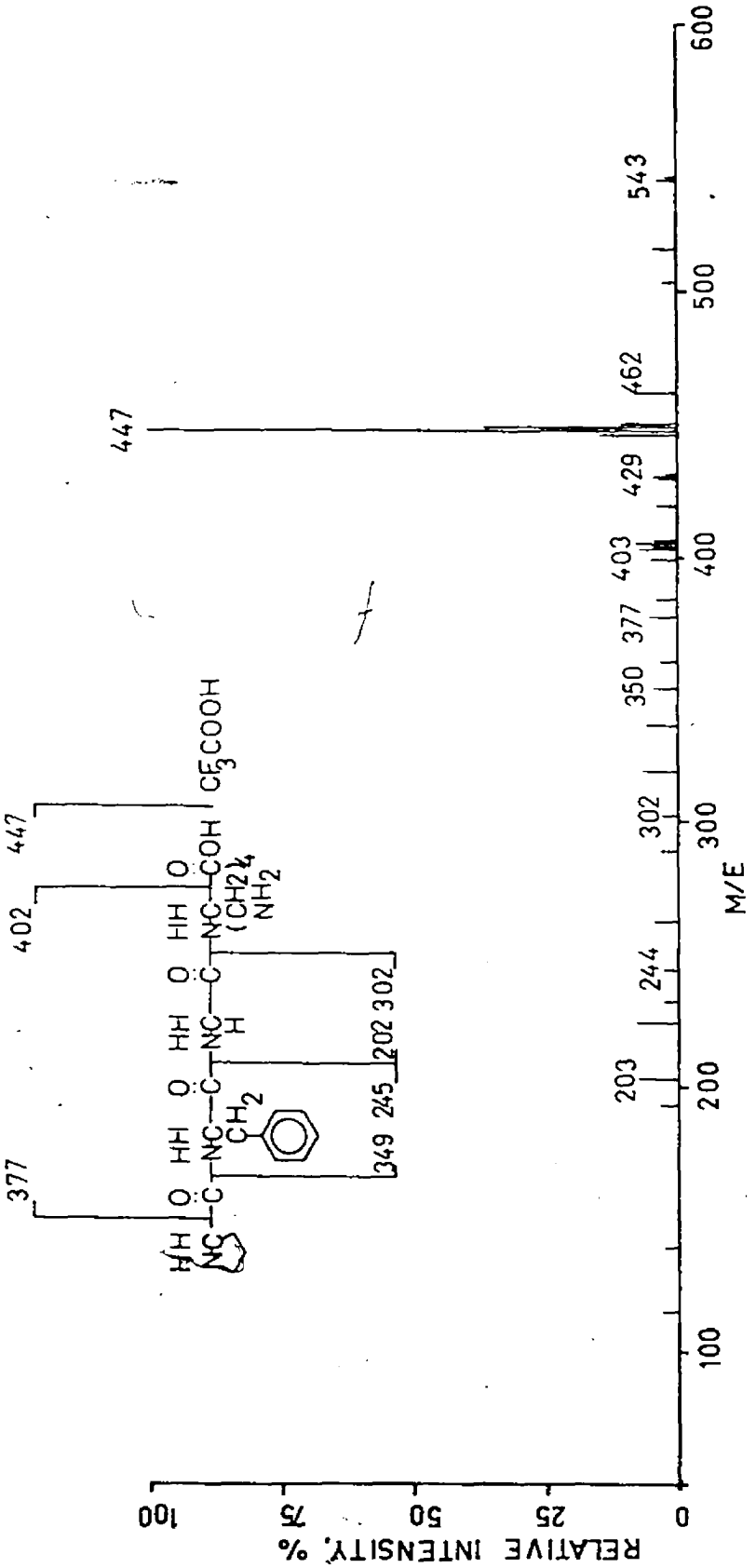


Fig. 11c : Field Desorption mass spectrum of H.ProPhe.Gly.Lys.OH. $COCF_3$ at 20-22mA.

FIGURE 11c

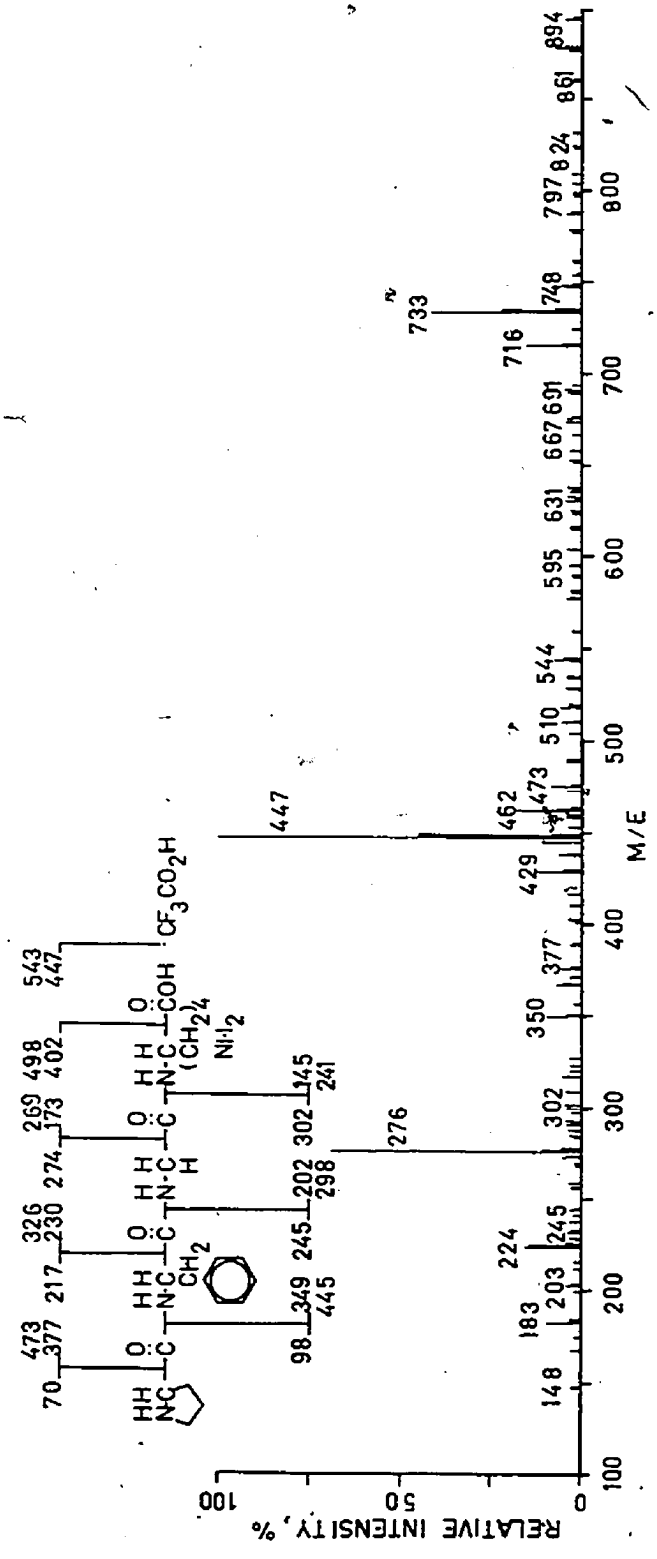
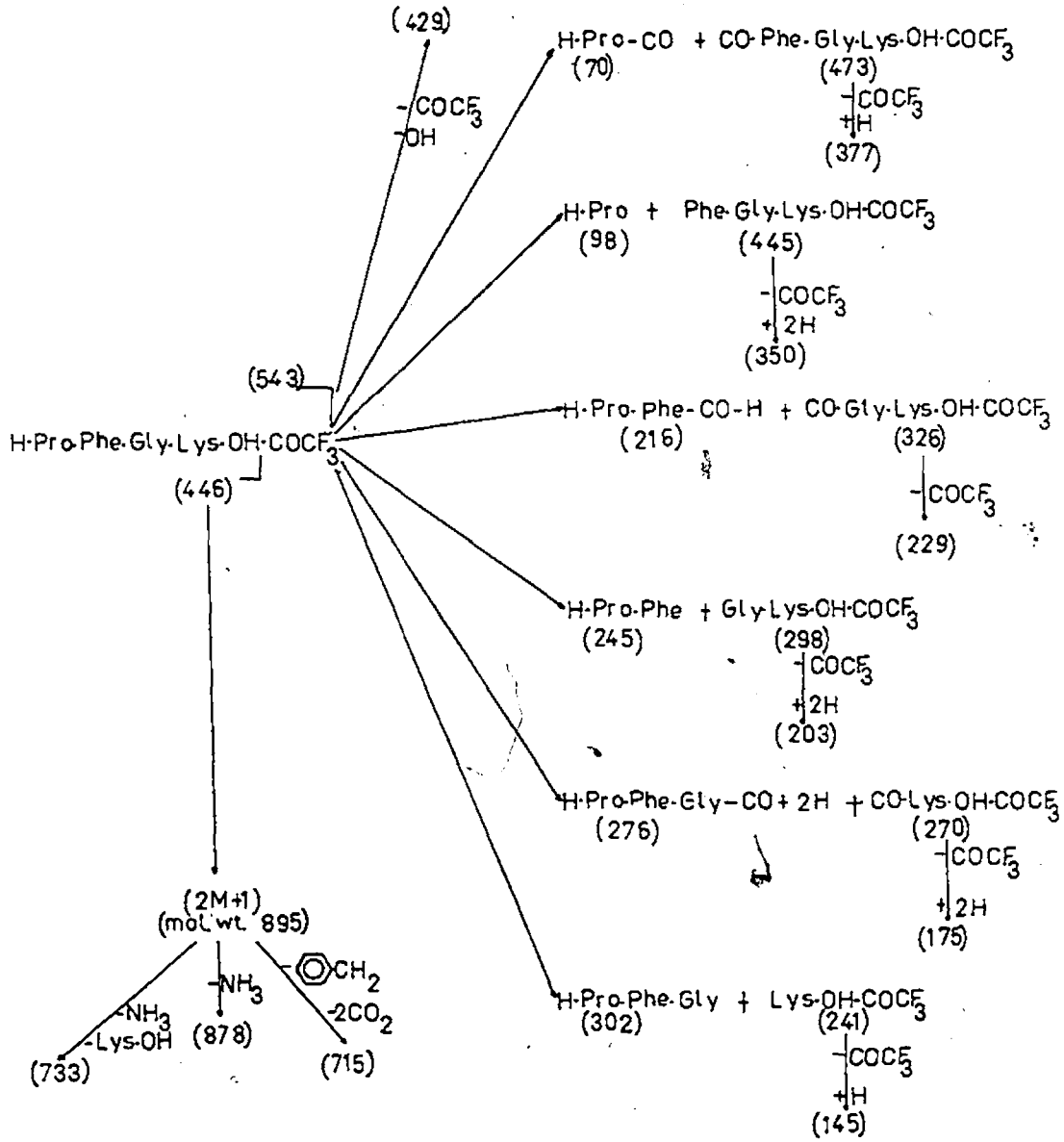
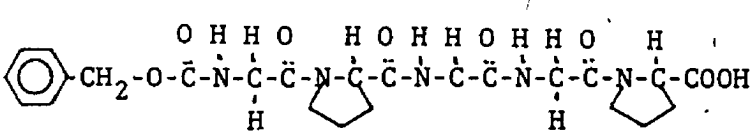


Fig. 12. Fragmentation scheme for tetrapeptide H.Pro.Phe. Gly.Lys.OH.$COCF_3$ showing possible products of decomposition.

FIGURE 12



3). N-CBZ-Gly.Pro.Leu.Gly.Pro.OH.



A cluster of peaks centered at m/e 574 ($[M+1]^+$) appear in the field desorption mass spectrum of the Cbz-pentapeptide, N-CBZ.Gly.Pro.Leu.Gly.Pro.OH (mol.wt.573.3) (Table XIII). The decomposition pattern is shown in Figure 13. Sequence peaks due to cleavage in the backbone of the peptide occur at m/e 467 (466+H), 439 (438+H), 382 (381+H), 261,284, 171,114,312 (311+H), 409 and 432 (431+H). Cleavage of the peptide backbone on either side of a carbonyl group with retention of the charge on the carboxyl terminal fragments was more frequent than retention of the charge on the amino terminal fragments. Carboxyl terminal sequence ions occur at m/e 114 (Pro.OH), 171 (Gly.Pro.OH), 284 (Leu.Gly.Pro.OH), 312 (CO.Leu.Gly.Pro.OH+H), 382 (Pro.Leu.Gly.Pro.OH+H), 409 (CO.Pro.Leu.Gly.Pro.OH), 439 (Gly.Pro.Leu.Gly.Pro.OH+H), and 467 (CO.Gly.Pro.Leu.Gly.Pro.OH+H). Amino terminal sequence ions were found at m/e 261 (Cbz.Gly.Pro-CO), 432 (Cbz.Gly.Pro.Leu.Gly.-CO+H) and 107 (Cbz-CO). The prominent peak at m/e 530 is due to loss of CO_2 from the $(M+1)^+$ ion.

The four ions appearing at m/e 591,620,663 and 681 have masses greater than the molecular ion of the Cbz-pentapeptide. These four peaks appear to arise from ions containing the peptide plus one or more molecules associated

with it. The peptide in the crystalline state contains one molecule of ethylacetate and one molecule of water (124). The peptide is then dissolved in ethanol: water (75:25) before application to the emitter. The peak at m/e 591 is assigned to a $(M.H_2O)^+$ ion but could also arise from a $(M+1.NH_3)^+$ ion which is formed by a rearrangement process. The peak at m/e 620 corresponds to the $[(M+1).CH_3CH_2OH)]^+$ ion while the peaks at 663 and 681 may arise from $[(M+2).CH_3CO_2CH_2CH_3)]^+$ and $[(M+2).CH_3CO_2CH_2CH_3.H_2O)]^+$ respectively.

Evidence on the origin of the ions at m/e 591, 620, 663 and 681 was provided by a hydrogen-deuterium exchange study. Field desorption mass spectrometry analysis was repeated using the same procedure as before but with the substitution of CD_3CD_2OD for CH_3CH_2OH and D_2O for H_2O. The results are shown in Table XIV. The molecular ion peak at m/e 573 $(MH_4)^+$ was increased in mass to correspond to the tetradeuterio species $(MD_4)^+$ at m/e 577 giving information on the number of exchangeable hydrogens in the peptide. The base peak at m/e 578 corresponds to the protonated tetradeuterio species $(MD_4+1)^+$. The peak at m/e 597 is ascribed to the tetradeuterio peptide (MD_4) plus D_2O (m/e 20). The ion at m/e 619 $(MH_4.CH_3CH_2OH)^+$ is increased in mass to correspond to a $(MD_4.CH_3CH_2OH)^+$ ion at m/e 623 (Table XIV). The ethanol appears to arise from ethylacetate rather than from solvent. The peaks at m/e 667 and 686 correspond to $[(MD_4+2).CH_3CO_2CH_2CH_3)]^+$ and $[(MD_4+1).D_2O.CH_3CO_2CH_2CH_3)]^+$ respectively.

Table XIII: Relative Ion Intensities (%) in f.d. mass spectrum of Cbz.Gly.Pro.Leu.Gly.Pro.OH (mol.wt.573.3)

m/e	Rel.int.	m/e	Rel.int.
107	8.5	440	5.2
114	3.7	467	7.0
171	6.0	530	50.5
260	3.0	531	36.0
261	4.5	532	11.2
262	3.1	556	7.4
284	4.0	557	5.0
285	3.5	573	49.0
286	4.2	574	100.0
306	4.4	575	61.3
307	5.1	576	25.1
312	3.1	581	7.0
374	3.6	591	5.7
381	3.4	619	6.6
382	5.6	620	6.6
396	4.0	621	5.4
397	8.0	663	14.3
409	3.4	664	8.2
426	7.8	665	5.2
432	4.1	671	4.3
438	4.0	680	4.2
439	9.2	681	7.2

Emitter heating current 20mA. Solvent: Ethanol: water (75:25). All ions of relative abundance greater than 3% are registered.

Fig. 13. Fragmentation scheme for N-labelled pentapeptide, N-CBZ.Gly.Pro.Leu.Gly.Pro.OH. showing possible products of decomposition.

FIGURE 13

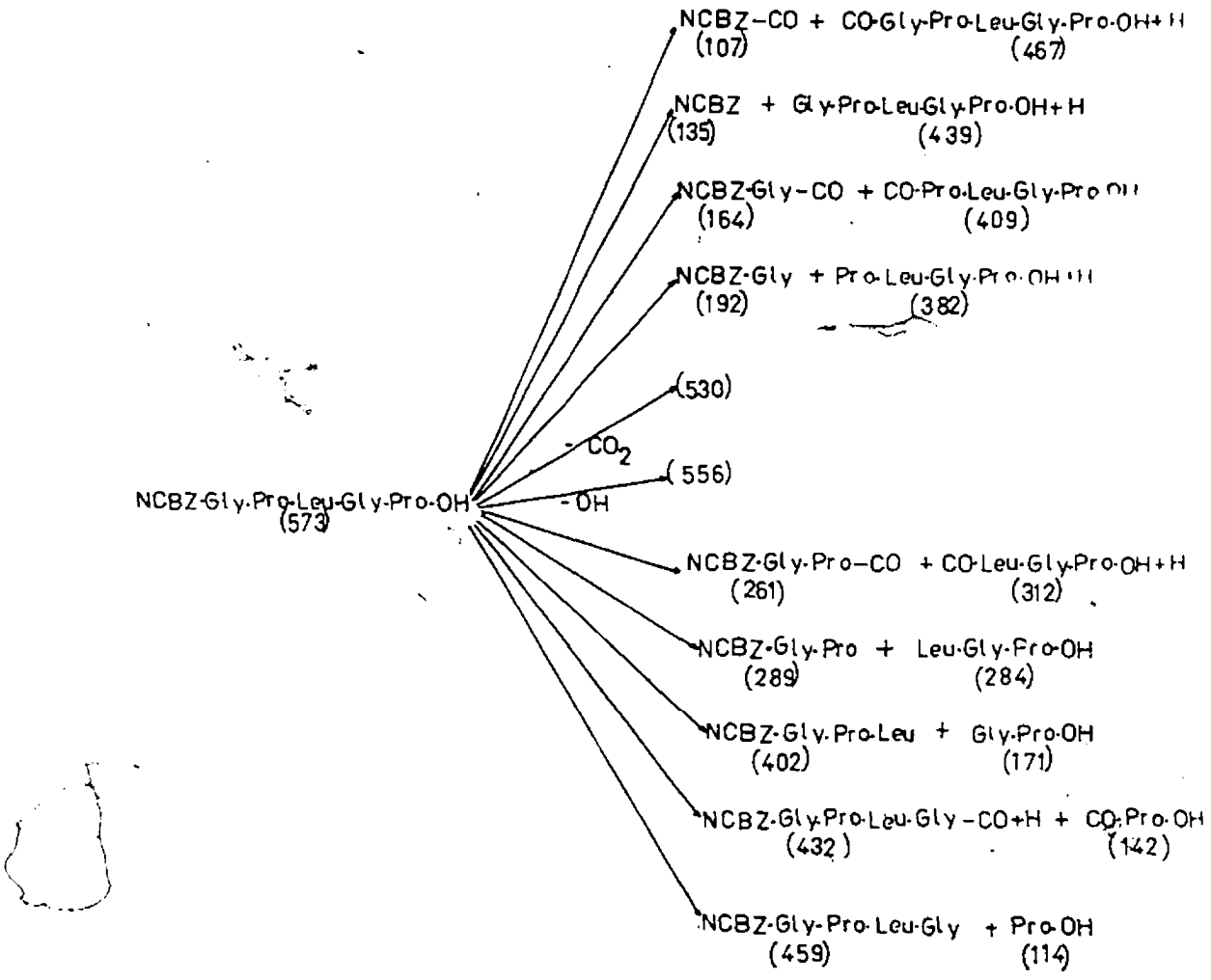


TABLE XIV: Comparison of f.d.m.s. of Cbz-Gly.Pro.Leu.Gly.Pro.OH in Ethanol: Water with Ethanol-d_6: Water -d_2

	Solvent			
	Ethanol: Water (75:25)		Ethanol-d_6: Water-d_2 (75:25)	
	m/e	R.I(%)	RI (%)	m/e
[a] $[MH_4]^+$	573	48	94	577 [b] $[MD_4]^+$
	574	100	100	578
	575	62	65	579
$[MH_4.H_2O]^+$	591	6	16	597 $[MD_4.D_2O]^+$
			14	598
$[MH_4.CH_3CH_2OH]^+$	619	7		
	620	7	7	622 $[MD_4.CH_3CH_2OH]^+$
	621	5	9	623
$[(MH_4+2).CH_3CO_2CH_2CH_3]^+$	663	14	9	667 $[(MD_4+2).CH_3CO_2CH_2CH_3]^+$
	664	8	6	668
	665	5		
$[(MH_4+1).CH_3CO_2CH_2CH_3.H_2O]^+$	680	4	4	686 $[(MD_4+1).D_2O.CH_3CO_2CH_2CH_3]$
	681			

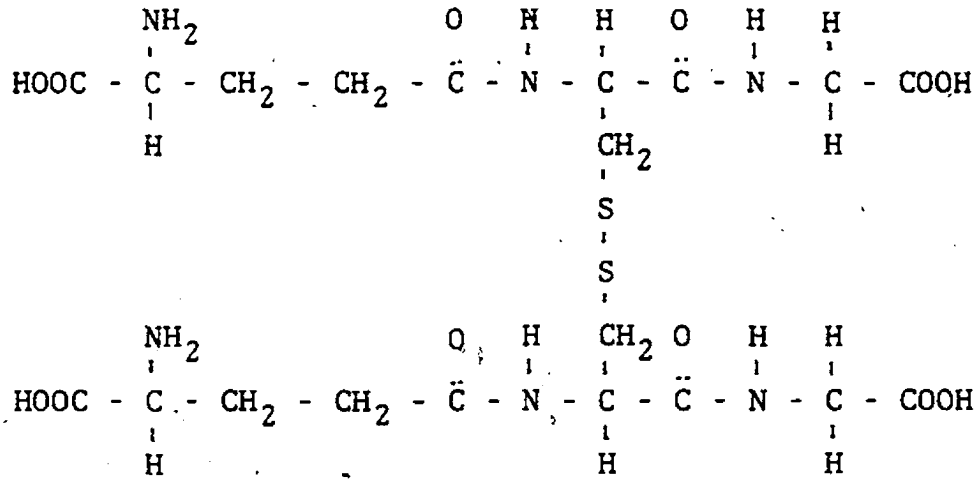
1) Threshold value of 4%. Other Other peaks 4% are 581 (7%) 671 (4%)

2) Threshold value of 4%. Other peaks 4% are at 588 (7%), 592(7%), 615 (7%) 619 (7%). 663 (6%) and 671 (6%).

a. MH_4 represents Cbz-pentapeptide with its four exchangeable protons.

b. MD_4 represents the Cbz-pentapeptide with the four exchangeable protons replaced by four deuteriums.

4). $(H.(HO\text{-}\gamma\text{-}Glu.)Cys.Gly.OH)_2$



The field desorption mass spectrum of the disulfide bridged peptide $(H.(HO\text{-}\gamma\text{-}Glu).Cys.Gly.OH)_2$ (mol.wt.612) gave m/e 307 ($[M/2+1]^+$ as the base peak (Table XV). The molecular ion peak was not observed. The mass spectrum was similar to that of H.(HO -γ- Glu).Cys.Gly.OH (mol.wt.307) as far as ions below the mass 307 were concerned. The fragmentation pattern of $(H.HO\text{-}\gamma\text{-}Glu).Cys.Gly.OH)_2$ is analogous to that of H.(HO -γ- Glu).Cys.Gly.OH (Figures 8 and 14).

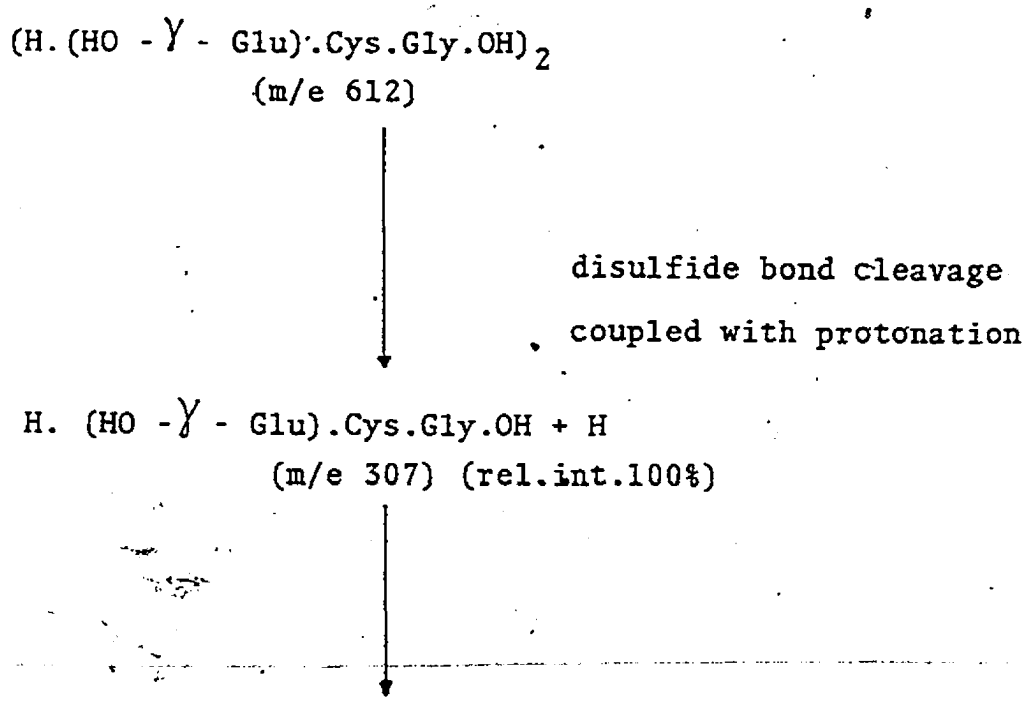
Table XV: Relative Ion Intensities (%) in f.d. mass spectrum of $(H.(HO-\gamma-Glu.)^{\gamma}.Cys.Gly.OH)_2$ (mol.wt.612.6).

m/e	Rel.int.	m/e	Rel.int.
145	4.2	275	10.2
146	4.4		
		277	7.6
177	5.1	278	5.2
204	5.3	285	7.6
		286	5.1
234	5.1		
		290	5.2
243	5.2		
244	5.9	298	6.8
245	5.1		
		307	100.0
250	4.2	308	8.5
253	4.3	324	20.5
		325	5.2
255	5.2		
		355	5.1
263	5.1		
265	4.2	374	4.6
270	5.1	402	4.2
		466	5.1

Emitter heating current – 22mA. Solvent: Distilled deionized water (with < 0.1 ppm sodium as sodium chloride). All ions of relative abundance greater than 4% are registered.

Fig. 14. Fragmentation scheme for underivatized hexapeptide $(H.(HO-\gamma - Glu).Cys.Gly.OH)_2$ showing possible products of decomposition.

FIGURE 14



E. NONAPEPTIDE (Bradykinin)

H.Arg.Pro.Pro.Gly.Phe.Ser.Pro.Phe.Arg.OH

The field desorption mass spectrum of the nonapeptide bradykinin is given in Figure 15. A series of overlapping peptide fragments shown in Figure 16 contain fragments from both the amino terminal and carboxyl terminal portions of the peptide. A molecular ion peak at m/e 1060 was obtained for the underivatized nonapeptide bradykinin (mol.wt.1059.6) (Figure 15). Sequence peaks due to the cleavage of the peptide backbone occur at m/e 351, 323, 736 and 527. Further sequence peaks are assigned at 255 (254 + H), 409 (408 + H), 556 (555 +H), 740 (739 + H), 806 (805 +H), 680 (679 + H), 533 (532 + H), and 321 (320 + H). Sequence ions assigned to fragments containing two added protons occur at m/e 419 (417 + 2H), 507 (505 + 2H) and 175 (173 + 2H) while a sequence ion of m/e 199 appears to originate from a (201-2H) ion. Amino terminal sequence ions are found at m/e 255 (H.Arg.Pro+H), 323 (H.Arg.Pro.Pro.Gly.+H), 527 (H.Arg.Pro.Pro.Gly.Phe-CO), 556 (H.Arg.Pro.Pro.Gly.Phe.+H), and 740 (H.Arg.Pro.Pro.Gly.Phe.Ser.Pro.+H).

Carboxyl terminal sequence ions appear at m/e 175 (Arg.OH + 2H), 199 (CO.Arg.OH -2H), 321 (Phe.Arg.OH + H), 419 (Pro.Phe.Arg.OH + 2H), 507 (Ser.Pro.Phe.Arg.OH + 2H), 533 (CO.Ser.Pro.Phe.Arg.OH + H), 680 (CO.Phe.Ser.Pro.Phe.Arg.OH+H), 736 (CO.Gly.Phe.Ser.Pro.Phe.Arg.OH), 806 (Pro.Gly.Phe.Ser.Pro.Phe.Arg.OH + H) (Figure 16). The field desorption mass spectrum

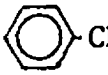
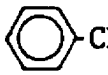
exhibits mass peaks which may arise from the cleavage of amino acid side chains. The peaks at m/e 777 and 789 arise from the expulsion of a portion of the arginine sidechain from the fragment CO.Pro.Gly.Phe.Ser.Pro.Phe.Arg.OH (mol.wt.833). The peak at m/e 777 is assigned to the fragment (Pro.Gly.Phe. Ser.Pro.Phe.Arg.)+CO–$(NH_2$ -C(NH)-NH)+2H$)^+$ and at m/e 789 assigned to the fragment ((Pro.Gly.Phe.Ser.Pro.Phe.Arg.OH) +CO–$(NH_2$-C=NH)–H$)^+$. Similarly the peaks at m/e 573 and 608 are assigned to the fragments [(H.Arg.Pro.Pro.GlyPhe.Ser) –CO–$(NH_2$-C=NH)+2H$]^+$ and ((Phe.Ser.Pro.Phe.Arg.OH)–(NH-C=NH))$^+$ respectively. Loss of a benzyl group from pheylalanine occurs in the fragments ((Pro.Gly.Phe.Ser.Pro.Phe.Arg.OH)-( -CH_2) +2H$)^+$ at m/e 716 and ((Ser.Pro.Phe.Arg.OH)-( -CH_2)+CO+2H$)^+$ at m/e 443. The peak at m/e 517 may arise from the expulsion of a portion of the arginine side chain (NH_2-C=NH) and a benzyl group  -CH_2 from phenylalanine from the fragment (Phe.Ser.Pro.Phe.Arg.OH) (mol.wt. 651).

Fig. 15 : Field Desorption mass spectrum of Bradykinin.

FIGURE 15

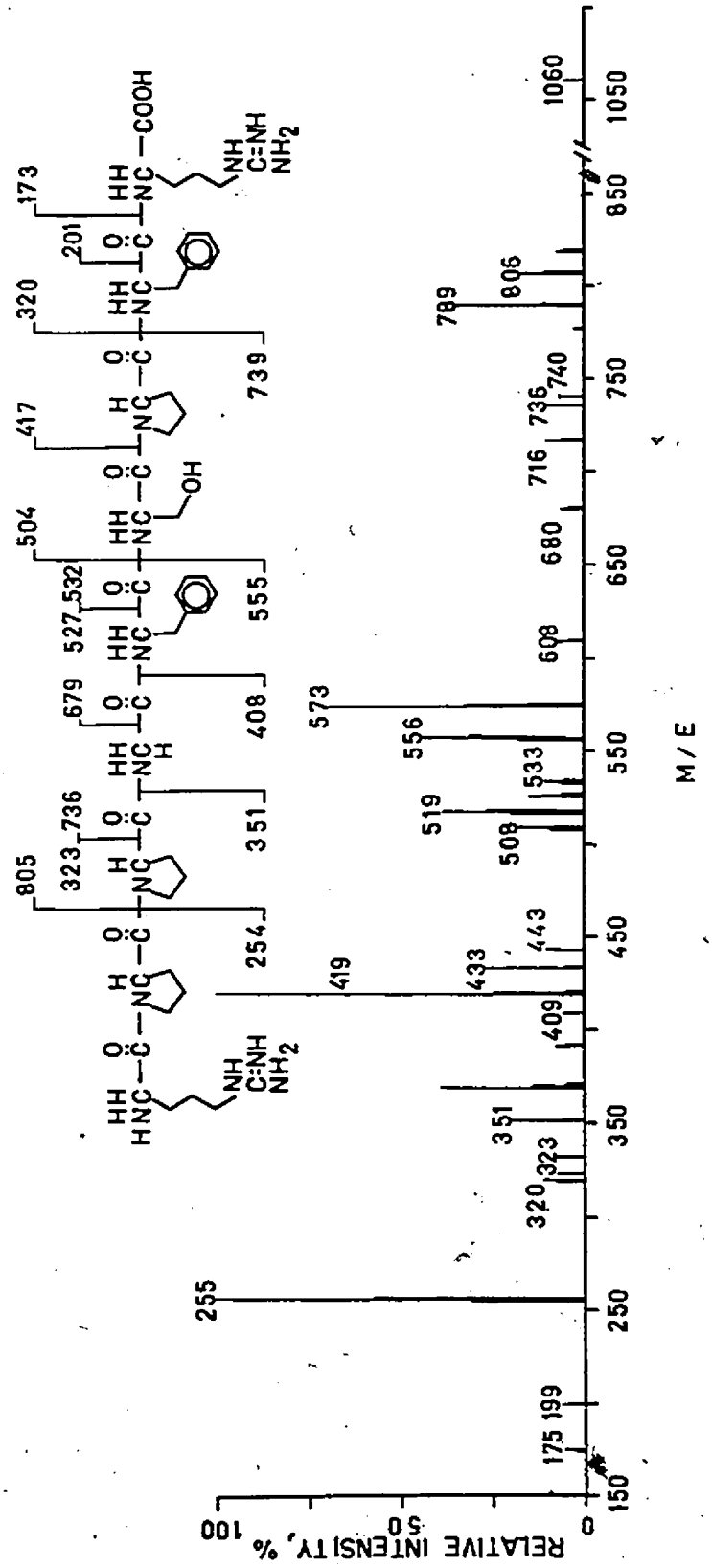
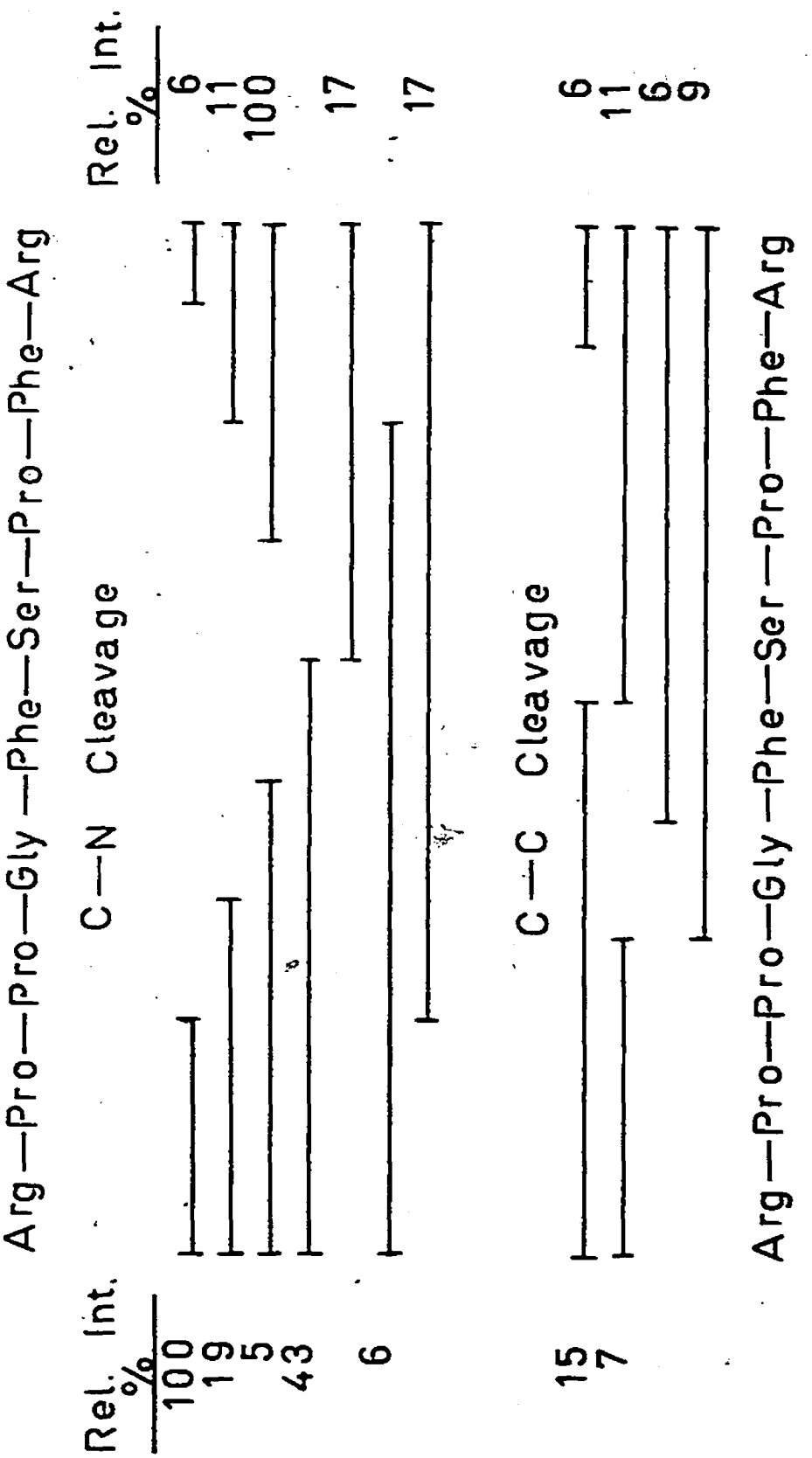


Fig. 16 : Sequence fragments obtained from the f.d. mass spectrum of Bradykinin.

FIGURE 16



F. MIXTURES

For the investigation of peptide mixtures using field desorption mass spectrometry it is necessary to run several mass spectra at different emitter heating currents. This study requires that molecular ions of each component of a mixture be present at one or more emitter heating currents. Interpretation is effected by comparing the spectra obtained at the different emitter heating currents and looking for changes in peak intensities as a component of a mixture becomes either more or less volatile relative to the others.

Two binary mixtures H.Phe.Val.OH (mol.wt.264), H.Gly.Ile.OH (mol.wt.188) and H.Pro.Leu.Gly.NH_2 (mol.wt.284), H.Met.Ala.Ser.OH (mol.wt.307) and a mixture containing three dipeptides, H.Phe.Val.OH (mol.wt.264) H.Gly.Ile.OH (mol wt.188) and H.His.Lys.OH (mol.wt.283) were studied.

1). MIXTURE 1

A mixture containing the two dipeptides H.Gly.Ile.OH (mol.wt.188) and H.Phe.Val.OH (mol.wt.264) was studied by field desorption mass spectrometry at emitter heating currents of 18mA and 20mA. The spectra are given in Table XVI. Table XVII presents molecular ion information from Table XVI. The component that is more abundant at the lower heating current (18mA) showed a base peak at m/e 265 which is consistent with the dipeptide H.Phe.Val.OH (mol.wt.264). The second component which gives rise to ions of increased abundance at the heating current (20mA) is the dipeptide H.Gly.Ile.OH (mol.wt.188) (Table XVII).

Table XVI: Mixture 1: Relative Intensities (%) of ions in the f.d. mass spectra of a mixture of H.Phe Val.OH (mol.wt.264) and H.Gly.Ile.OH (mol.wt.188 at different emitter heating currents

18 mA		20 mA	
m/e	Rel.int.(%)	m/e	Rel.int.(%)
30	12.5	30	7.7
46	20.3	60	6.1
107	9.1	75	5.6
120	23.3	79	7.2
129	15.8	85	9.7
130	9.1	92	10.7
144	37.4	93	11.2
145	21.1		
149	14.0	115	5.6
150	7.5	121	23.5
158	5.1	129	7.2
170	19.4	130	22.0
171	19.0	131	7.2
173	11.0	142	8.7
174	21.5	143	14.8
188	39.8	144	55.2
189	15.3	145	85.9
208	12.0	146	59.3
209	21.2	147	9.2
220	31.6	172	11.7
247	51.1	174	6.1
248	30.9	175	8.2
249	14.0	176	5.1
250	12.1	187	16.8
		188	100.0
		189	16.8

m/e	Rel.int.(%)	m/e	Rel.int.(%)
264	84.1	220	9.7
265	100.0	221	10.2
266	24.3		
		243	5.1
		244	7.2
		247	100.0
		248	75.2
		249	21.5
		257	5.6
		264	12.2
		265	64.3
		266	19.4
		267	5.1

Solvent: Ethanol: Water (75:25). Threshold Value 5%.

Table XVII: Molecular ion information from f.d. mass spectra data on mixture 1

Peptide	EMITTER HEATING CURRENTS 18mA Rel.int.(%) $(M)^+$	18mA Rel.int.(%) $(M+1)^+$	20mA Rel.int.(%) $(M)^+$	20mA Rel.int.(%) $(M+1)^+$
H.Phe.Val.OH (mol.wt.264)	84.1	100.0	12.2	64.3
H.Gly.Ile.OH (mol.wt.188)	37.8	15.3	100.0	16.8

2). MIXTURE 2

This mixture consisting of equimolar amounts of H.Gly.Ile.OH (mol.wt.188), H.Phe.Val.OH (mol.wt.264) and H.His.Lys.OH (mol.wt.283) was studied at three emitter heating currents, 16.5mA, 18mA and 20mA. The spectra are presented in Table XVIII. Molecular ion information from Table XVIII is given in Table XIX. A component H.His.Lys.OH (mol.wt.283) would explain the base peak at m/e 284 (16.5mA heating current) (Table XIX). The base peak at m/e 188 (at 18mA heating current) would indicate the dipeptide H.Gly.Ile.OH (mol.wt.188) while the component H.Phe.Val.OH (mol.wt.264) explains the base peak at m/e 264 (20mA heating current) (Table XVIII). With increasing heating current some components were almost lost completely (Table XVIII).

Table XVIII: Mixture 2: Relative Ion Intensities (%) in f.d. mass spectra of a mixture of H.Gly.Ile.OH (mol.wt.188), H.Phe.Val.OH (mol.wt.264) and H.His.Lys.OH (mol.wt.283) at different emitter heating currents.

16.5mA		18mA		20mA	
m/e	Rel.int.(%)	m/e	Rel.int.(%)	m/e	Rel.int.(%)
29	23.7	30	4.3	188	5.1
30	22.8	75	5.1	247	14.6
43	31.5				
44	10.5			264	8.1
45	67.0	92	5.2	265	100.0
46	65.0			266	24.8
		100	5.9	267	4.4
91	14.3				
92	6.7	132	10.2	279	8.1
93	5.7	133	8.5	284	4.4
100	8.6	141	13.5		
101	6.7	143	6.8		
114	4.8	146	6.8		
115	6.7	170	9.4		
118	10.5	172	5.1		
119	4.8	173	4.3		
141	6.7	187	4.3		
142	15.2	188	100.0		
143	4.8	189	10.2		
		236	5.9		
170	6.7	238	6.8		
172	4.8				
		245	8.5		
187	10.5	246	6.8		
188	36.1	247	5.9		
189	8.6				

16.5mA		18mA		20mA	
m/e	Rel.int.(%)	m/e	Rel.int.(%)	m/e	Rel.int.(%)
264	15.0	264	40.0		
265	41.0	265	56.0		
266	9.6	266	14.2		
283	89.5	269	9.4		
284	100.0	284	48.5		
285	85.0	285	8.5		
		296	4.3		
		297	5.9		

Solvent; Ethanol: Water (75:25). Threshold value 4%.

TABLE XIX: Molecular ion information from f.d. mass spectra data on Mixture 2.

Peptide	EMITTER HEATING CURRENTS					
	16mA Rel.int.(%)		18mA Rel.int.(%)		20mA Rel.int.(%)	
	$[M]^+$	$[M+1]^+$	$[M]^+$	$[M+1]^+$	$[M]^+$	$[M+1]^+$
H.Gly.Ile.OH (mol.wt.188)	36.1	8.6	100.0	10.2	5.1	-
H.Phe.Val.OH (mol.wt.264)	15.2	41.0	40.3	56.0	8.1	100.0
H.His.Lys.OH (mol.wt.283)	89.5	100.0	48.5	8.5	4.4	-

3). MIXTURE 3

A mixture containing equimolar amounts of the two tripeptides, H.Pro.Leu.Gly.NH_2 (mol.wt. 284) and H.Met.Ala Ser.OH (mol.wt. 307) was studied at three emitter heating currents, 15 mA, 16.5 mA and 18 mA. Table XX presents the spectra and Table XXI shows the molecular ion information from Table XX. For sequence determination the amino-terminal and the carboxyl-terminal amino acid residues must be found by comparing the spectra at the different emitter heating currents. In addition overlapping dipeptide fragments and the molecular ions must be present.

Examination of the spectrum obtained at 15 mA showed a base peak at m/e 285 (Table XX). Amino-terminal amino acids are indicated by the peaks at m/e 98 (H.Pro), m/e 70 (H.Pro-CO), and the peaks at m/e 132 (H.Met) and m/e 103 (H.Met-CO-H). Carboxyl-terminal amino acids are indicated by the peaks at m/e 74 (Gly.NH_2+H), 132 (CO.Ser.OH) and 103 (Ser.OH-H). Beyond m/e 132 the next ion corresponding to an amino acid difference is at m/e 186 which indicates a Leu.Gly.NH_2 sequence while the peak at m/e 215 indicates a (H.Met.Leu-CO-2H) sequence. At 16.5 mA the base peak is at m/e 70, again indicating an amino-terminal proline. Two intense peaks were observed at m/e 285 (95.6% rel.int.) and m/e 308 (96.5% rel.int.) The peaks at m/e 132 and 104 place methionine at the amino-terminal

while carboxyl-terminal amino acids are indicated by peaks at m/e 74 (Gly.NH_2+H), 132 (CO.Ser.OH) and 104 (Ser.OH). Beyond m/e 132 the next ion corresponding to an amino acid mass difference is at m/e 203 which indicates a H.Met.Ala sequence and m/e 202 which corresponds to a (H.Pro.Met.-CO+H) sequence. Again the peak at m/e 215 indicates the sequence (H.Met.Leu-CO-2H). Carboxyl-terminal dipeptides are indicated by the peaks at m/e 215 (Leu.Ser.OH-2H) and m/e 143 (Ala.Gly.NH_2-H). At 18 mA the base peak occurred at m/e 308 (Table XX).

Following the requirements for the sequence determination of tripeptides that the amino-terminal and the carboxyl-terminal amino acid residues must be found in addition to overlapping dipeptide fragements and that the molecular ion must occur, nine possible sequences are proposed (Table XXII).

On the basis of the spectra obtained at the three emitter heating currents the only component consistent with a molecular ion at m/e 285 is H+Pro +Leu+Gly+NH_2+H(mol.wt.284). Similarly the only component consistent with the molecular ion at m/e 308 is H+Met+Ala +Ser +OH+H (mol.wt. 307) (Table XXI). No sequence peaks were found which would allow a unique sequence determination of these two peptides.

Table XX: Mixture 3. Relative ion intensities (%) in f.d. mass spectra of a mixture of H.Pro.Leu.Gly.NH_2 (mol.wt.284) and H.Met.Ala.Ser.OH (mol.wt.307) at different emitter heating currents.

15 mA		16.5 mA		18 mA	
m/e	Rel.int.(%)	m/e	Rel.int.(%)	m/e	Rel.int.(%)
				74	20.3
44	8.0	43	30.5	102	31.0
		44	21.7	104	50.0
70	63.5				
		70	100.0		
71	11.1	71	12.3	113	37.5
74	6.3	74	18.7	144	50.1
75	6.2				
		85	26.1	152	37.8
93	11.1			153	31.2
		101	17.4		
98	12.7	102	26.1	202	25.0
				203	37.5
102	11.2	104	21.7		
103	9.5			215	31.6
		132	52.2		
132	11.3			210	31.3
		136	21.9		
				253	37.4
186	6.4	143	26.3		
187	6.4				
				267	25.0
		146	26.0	268	31.2
215	11.3				
		202	21.4	285	35.0
242	8.0	203	34.9	286	11.2
267	6.5				
		215	21.7	306	18.8
				307	87.5
283	12.7	267	21.3	308	100.0
284	58.9			309	48.7
285	100.0	283	21.7		
286	19.1	284	30.4		

15 mA		16.5 mA		18 mA	
m/e	Rel.int.(%)	m/e	Rel.int.(%)	m/e	Rel.int.(%)
		285	95.6		
		286	21.1		
307	4.8				
308	6.4	307	48.0		
		308	96.5		
		309	26.1		

Table XXI: Molecular ion information from f.d. mass spectra data on mixture 3.

	EMITTER HEATING CURRENTS					
	15mA		16.5mA		18mA	
	Rel.int.(%)		Rel.int.(%)		Rel.int.(%)	
Peptide	$(M)^+$	$(M+1)^+$	$(M)^+$	$(M+1)^+$	$(M)^+$	$(M+1)^+$
H.Pro.Leu.Gly.NH_2 (mol.wt.283)	58.9	100.0	31.4	95.6	-	35.0
H.Met.Ala.Ser.OH (mol.wt.307)	4.8	6.4	48.0	96.5	87.5	100.0

Table XXII: Postulated Sequences for Mixture 3

Sequence	Sequence ions	Molecular ion $(M)^+$
H.Pro.Met.Ser.OH	70/98, 202, 134/104	333
H.Pro.Met.Gly.NH_2	70/98, 202, 203, 102/74	303
H.Pro.Met.Leu.OH	70/98, 202, 132	359
H.Pro.Leu.Gly.NH_2	70/98, 202/186, 102/74	284
H.Met.Leu.Ser.OH	104/132, 215,132/104	349
H.Met.Leu.Gly.NH_2	104/132, 215/186, 102/74	319
H.Met.Ala.Set.OH	104/132, 203,132/104	307
H.Met.Ala.Gly.NH_2	104/132, 203,145, 102/74	277
H.Met.Ala.Leu.OH	104/132, 203,132	333

G. Removal of Sodium

Figures 17 to 20 give the results of attempts to remove inorganic impurities (sodium and potassium) from peptide samples. Three different types of materials were employed for the removal of impurities: gel filtration using Bio Gel P-2, ion retardation using Bio Rad AG 11 A8 and ion exchange using Amberlite IR 120 (H^+ form). None of the three materials could render the peptide samples free of sodium and/or potassium. Desorption of the peptides treated with either Bio Gel P-2 or Bio Rad AG 11 A8 was found to be smoother and at a relatively lower emitter heating current than when treated with Amberlite IR 120 (H^+form).

Effect of Sodium on f.d.m.s. of H.Pro.Phe.Gly.Lys.OH.$COCF_3$ (mol.wt. 543)

In the study of the effect of sodium on the field desorption mass spectrum of tetrapeptide H.Pro.Phe.Gly.Lys.OH.$COCF_3$(mol.wt.543) the salt (sodium chloride) and the peptide were adsorbed from an ethanol (75%) solution on the emitter using the dipping technique (89). The mixtures were made up by preparing a series of ethanolic (75%) solution of 1.1μM

peptide containing sodium at 4.3×10^{-4} μM, 4.3×10^{-3} μM, 4.3×10^{-2} μM and 4.3×10^{-1} μM. The total spectra in the molecular ion region are given in Table XXIII. For the range of sodium (0 to 4.3×10^{-1} μM) studied the base peak was found at m/e 447 (Peptide. $COCF_3$ - $COCF_3$ +H). The range of emitter heating current used was found to be relatively small (Δi 2mA).

Addition of sodium (4.3×10^{-4} to 4.3×10^{-1} μM) to the peptide produced two relatively intense ions corresponding to a $(M+Na)^+$ ion and a $(M+2Na)^+$ ion.

Table XXIII

Effect of Sodium on f.d.m.s. of H.Pro.Phe.Gly.Lys.OH.$COCF_3$ (M.$COCF_3$) [a]

	Amount of Peptide 1.1μM				
	0 μM Sodium	4.3×10^{-4} μM Sodium	4.3×10^{-3} μM Sodium	4.3×10^{-2} μM Sodium	4.3×10^{-1} μM Sodium
m/e	Rel.int.	Rel.int.	Rel.int.	Rel.int.	Rel.int.
447($[M]^+$)	100.0	100.0	100.0	100.0	100.0
M + Na	2.5	45.8	9.5	38.5	73.8
M + 2Na	-	6.3	2.1	2.9	11.5

[a] The first spectrum taken after focusing is presented.

Fig. 17 : Desalting of Angiotensin I on AG 11 A8 column.(0.9 x 42 cm).

Amount of peptide on the column: 7.7×10^{-1} µM .

—o— peptide.

—●— sodium.

FIGURE 17

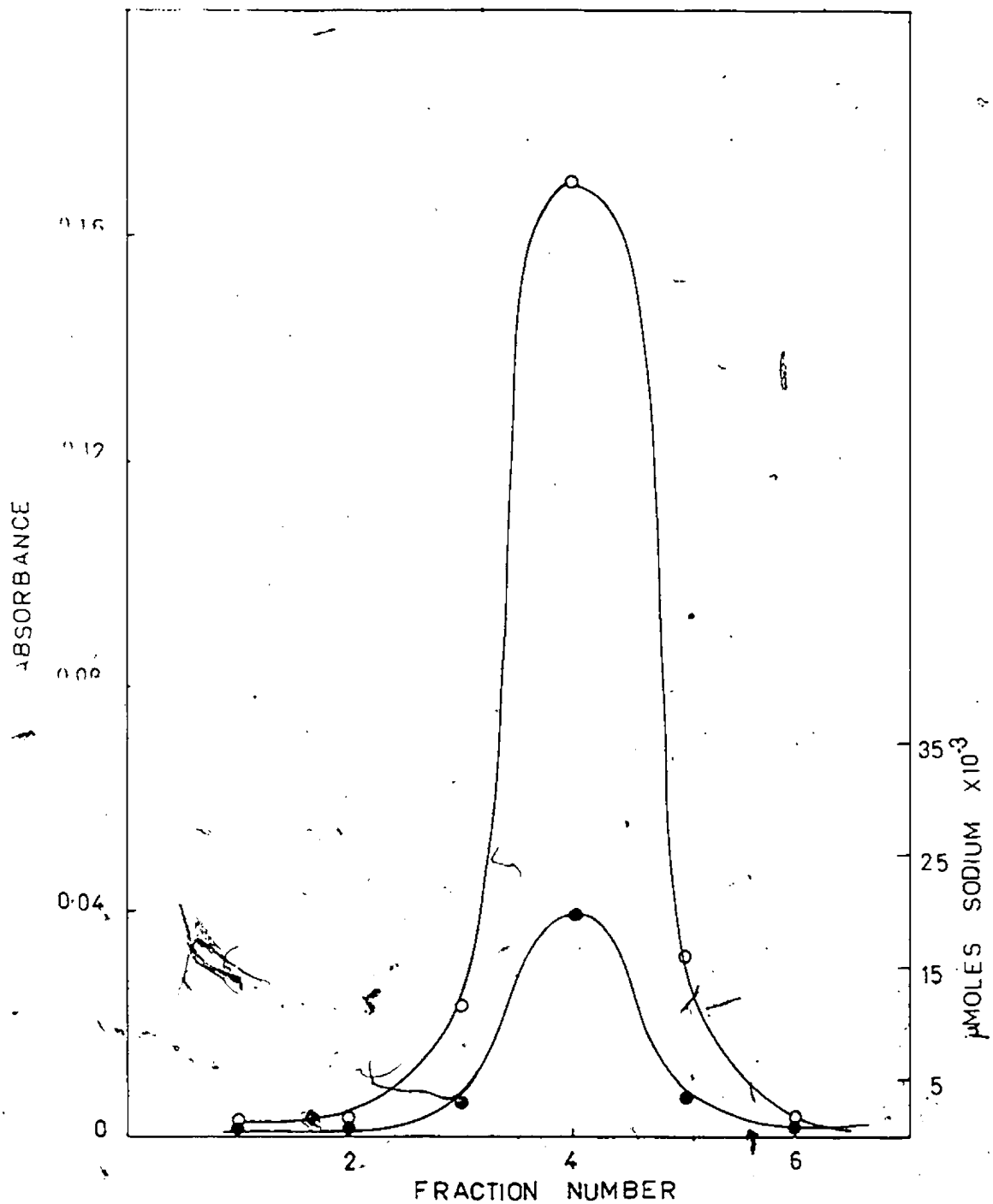


Fig. 18 : Desalting of Glutathione(oxidized) on AG 11 A8 column(0.9 x 42 cm).
Amount of peptide on the column: 1.03×10^{-2} mM + 1.74×10^{-2} mM sodium .
—o— peptide.
—●— sodium.

FIGURE 18

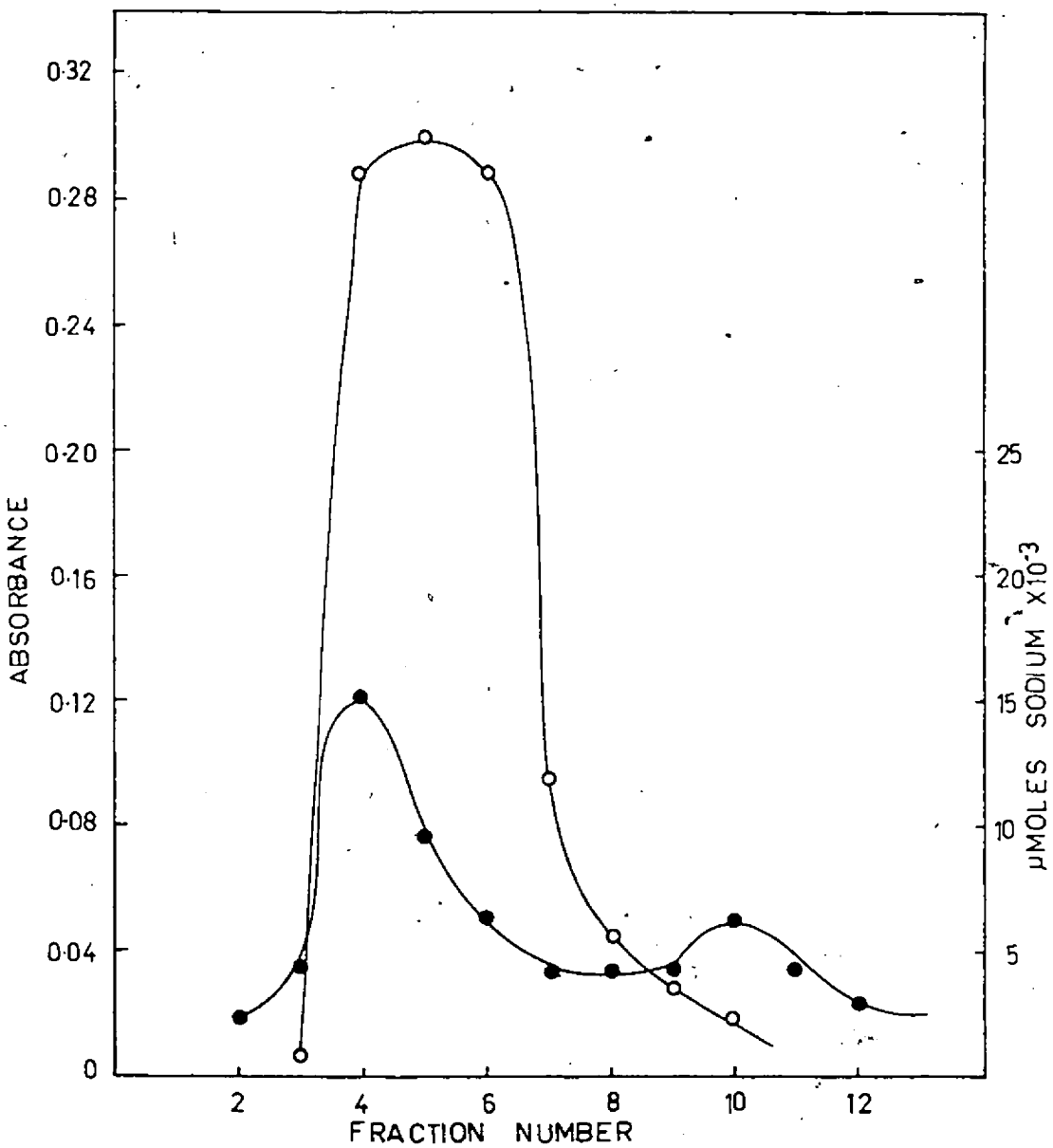


Fig. 19 : Desalting of Glutathione (oxidized) on Amberlite IR 120 (hydrogen form) column (0.9 x 50 cm).
Amount of peptide on the column: 3.97×10^{-2} mM + 1.74×10^{-2} mM.
—o— peptide.
—●— sodium.

FIGURE 19

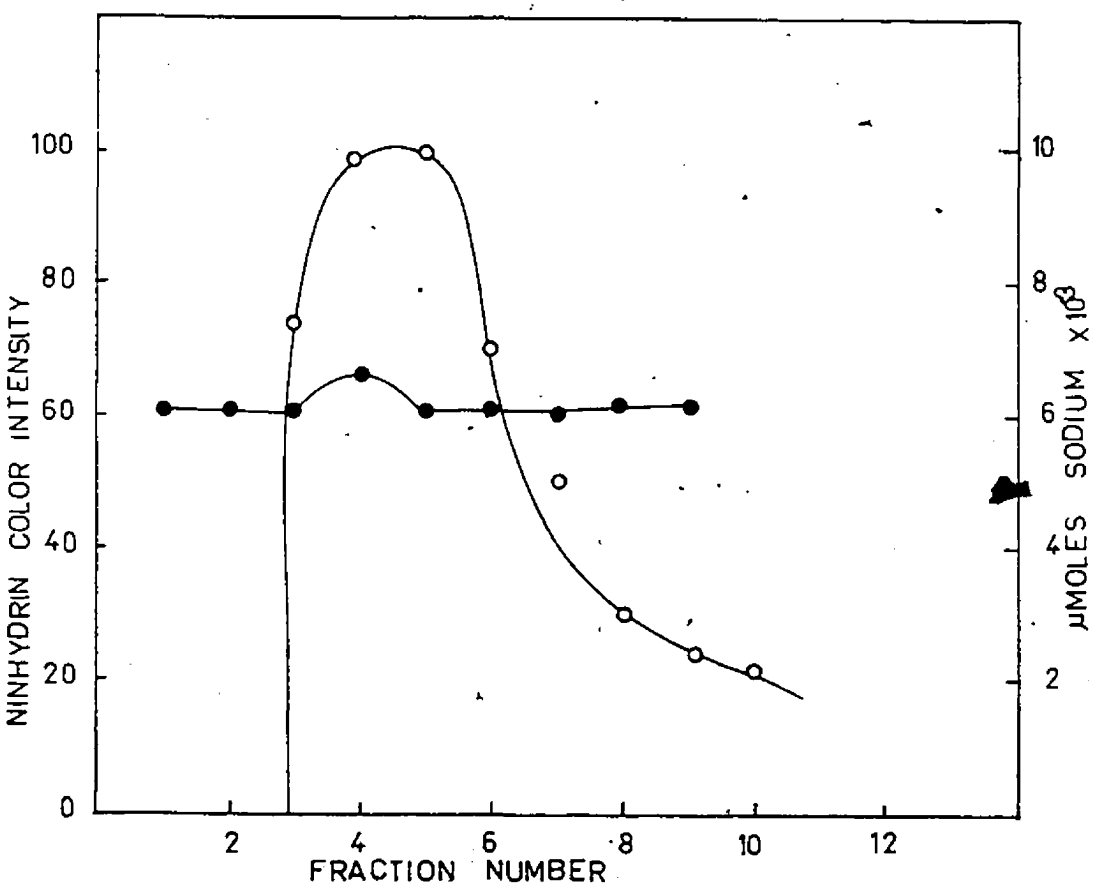


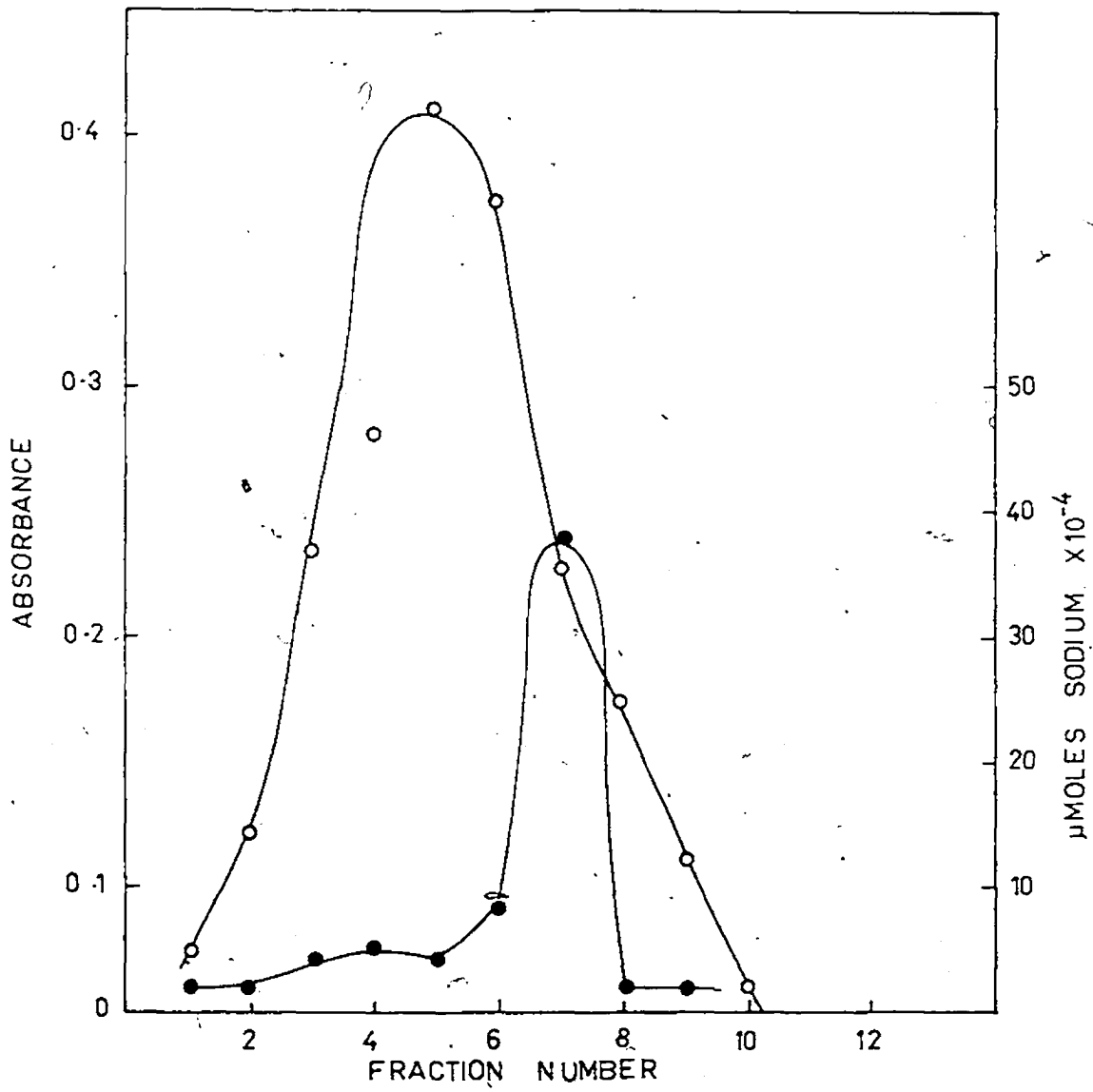
Fig. 20 : Desalting of Angiotensin I on Bio Gel P-2 column (0.9 x 30 cm)

Amount of peptide on the column; 7.7×10^{-1} µM.

—o— peptide.

—●— sodium.

FIGURE 20



CHAPTER IV

DISCUSSION

In the framework of the field desorption mass spectrometry (f.d.m.s) investigation of oligopeptides, the spectra of a series of free amino acids need to be known. Studies in the areas of e.i.m.s. of free or esterified amino acids reveal that in general molecular ion information $[M]^+$ was often of low abundance (<1%, except for methionine, phenylanine and tryptophan) because of the facile loss of the carboxyl group (35,129-133). In c.i.m.s. the amino acids (except arginine and cystine) give spectra with relatively increased molecular ion intensity (134). The f.d.m.s. of the free amino acids investigated for the most part only confirm conclusions already reached by Winkler and Beckey (91). The f.d.m.s. mass spectra of the seven amino acids (lysine, phenylalanine, histidine, threonine, leucine, tyrosine and tryptophan) exhibit intense molecular ions ($[M]^+$ and/or $[M+1]^+$) and the only fragment ion (except for lysine which also losses NH_3) in all cases is formed by the loss of COOH from $[M]^+$ ion or $COOH_2$ from $[M+1]^+$ ion. At the best emitter heating current this was the total extent of fragmentation. Fragmentation, however, could be induced by using higher emitter currents;

on the other hand raising the emitter current also raises the rate of desorption and the emission current decreases rapidly during the mass scan. The range of currents between the beginning of desorption and the complete removal of the samples is relatively larger (4-8 mA) than that reported by Winkler and Beckey (2-3 mA) for ten amino acids (glutamic acid, valine, methionine, proline, serine, aspartic acid, histidine, arginine, cystine) studied (91). One reason for this difference may be that the solvents used in the present study (0.1M acetic acid and 75% ethanol solution) promote smoother field desorption of the samples.

Rearrangement processes which are prevalent in the e.i.m.s. of amino acids and their derivatives are generally absent from the f.d.m.s. of free amino acids probably because of the even character of the ions. Also the possibility of transfer of Franck-Condon excitation energy from the ionized molecules to the anode surface or to neighboring molecule would tend to decrease fragmentation (135).

The application of conventional mass spectrometer techniques to sequencing of oligopeptide is limited by the various difficulties indicated in the Introduction, that is, the notoriously low volatility of this class of compounds and the complexity of their e.i. mass spectra. This problem has been attacked chemically in the case of conventional mass spectrometry of peptides by the introduction of suitable

protecting groups to reduce inter-molecular hydrogen bonding. A potential solution to the problem of volatility of peptides is found in the application of f.d.m.s. to peptides adsorbed on inert, high temperature activated emitter surfaces (62).

Sequence information in e.i.m.s. arise from simple cleavage of the bonds on either side of the carbonyl group of the peptide backbone (40,63,115). A similar cleavage pattern is seen in the oligopeptides investigated by f.d.m.s. Determination of the amino acid sequence of peptides necessitates the production of both amino-terminal and carboxyl-terminal sequence ions. With the substitution of the field desorption method for the more conventional electron impact method, the question of fragmentation (other than at the carbonyl group of the peptide backbone) prior or subsequent to ionization becomes less important. This is due to the "gentle" nature of the ionization process and the elimination of the need to vaporize the compound prior to ionization (89).

The determination of the sequence of the dipeptides H.Gly.Ile.OH and H.Phe.Val.OH is straight forward. Unprotected dipeptides should have five sequence determining ions in the mass spectra and indeed the low resolution f.d.mass spectrum of H.Gly.Ile.OH contains carboxyl-terminal sequence ions at m/e 130 and 159, amino terminal sequence ion at m/e 30 and the molecular ion at m/e 188. This allows the formulation of the compound as H.Gly.Ile.OH precluding absolutely the isomeric H.Ile.Gly.OH. Similarly, the unprotected dipeptide H.Phe.Val.OH contains a carboxyl terminal determining ion

at m/e 146, amino terminal sequence determing ions at m/e 148 and 120, and the quasi-molecular ion $[M+1]^+$ at m/e 265. Of the two possible sequences only the sequence H.Phe.Val.OH fits all the data.

Under e.i.m.s. conditions, the parent ion has a sufficient ion source residence time (10^{-6} sec) for rearrangements as well as simple cleavages to take place. In the f.d.m.s. mode, however, the ion life-time within the source is approximately 10^{-13} to 10^{-11}sec which is insufficient for rearrangement to occur after ionization. However, the f.d.m.s. of histidine dipeptides, H.His.Lys.OH, H.His.Ser.OH and H.His.Tyr.OH, show prominent normal peaks which may be due to rearrangement and/or multistep reactions (Scheme 1). Beckey et al (100) have reported the e.i.-fi-f.d. mass spectra of a dipeptide, H.Gly.Gly.OH which exhibits normal prominent peaks due to rearrangements and multistep reactions in all three ionization modes. Sequence-characteristic rearrangement peaks in f.i. spectra of some benzyloxycarbonyl and tert-butyloxycarbonyl derivatives of simple peptides retain their importance relative to simple cleavage peaks as compared with e.i. spectra (61).

Signals of smaller intensities indicating multistep processes such as association and rearrangement reactions similar to e.i.m.s. have been reported for f.d.m.s. of sultams (98). Prominent normal peaks due to rearrangement reactions have also been reported for the f.d.m.s. of carbamates by Rouse et al (128). The rearrangement products seen in the

histidine dipeptides may be the result of initial thermal and/or field-induced surface reactions between the molecules adsorbed on the emitter surface and may also be due to the lower activation energies for the rearrangement processes compared with the direct bond fission. The cyclization of the histidine dipeptides on f.d.m.s. does not detract significantlly from the usefulness of the mass spectra. Accurate conclusions concerning the structure and identity of the samples are possible. However, knowledge that cyclization has occurred is imperative for an accurate and complete explanation of the spectra.

Milne et al (65) and Senn et al (115) suggested that in the e.i.m.s. of histidine peptides bimolecular reactions can occur on or near the probe producing inter-molecular methylation of the ring nitrogen of histidine residues. Sequence information ions may be accompanied by four mass units to lower mass ($M + CH_2-H_2O$). In the f.d.m.s. of underivatized histidine dipeptides such N-methylation and dehydration could not occur because there are no free methyl groups in the peptides studied to bring about transmethylation.

The f.d. mass spectra of tripeptides display intense molecular ions ($[M]^+$ and/or $[M+1]^+$). With all the tripeptides investigated field induced hydrogen addition to the peptide is followed by elimination of small stable molecules from the quasi-molecular $[M+1]^+$ ion as observed for sugars (89), chlorinated hydrocarbons (95) and sultams (98). Furthermore, hydrogen addition to the peptides lead to direct bond

ruptures in the peptides yielding easily intelligible and complete sequence information. Over lapping dipeptide fragments as well as amino terminal and carboxyl terminal amino acids are observed. If the tripeptide H.Pro.Leu.Gly.NH_2 were treated as an unknown, the ions of m/e 285 coupled with an independent amino acid analysis would establish the molecule as a tripeptide. The peak at m/e 73 and 101 indicate glycine is at the carboxyl terminal position and the peaks at m/e 70, 214 and 186 place proline at the amino terminal position. The peaks at m/e 186 and 214 indicate that glycine and leucine are adjacent. Only the sequence H.Pro.Leu.Gly.NH_2 fits all the data (Table IX). The mode of fragmentation of H.(HO -Y- Glu.).Cys.Gly.OH (Fig. 8) and H.Met.Ala.Ser.OH (Fig.9) is primarily characterized by cleavage of peptide bonds. The spectra are dominated by peaks reflecting the sequence of the tripeptides.

The tripeptide H.Met.Ala.Ser.OH illustrates a problem arising from isobaric masses of combinations of amino acids. The determination of the sequence by low resolution f.d.m.s. would be difficult without independent supplementary information. Identifications in this particular case were confirmed by a study of isotopic peaks of sulfur in the ions at m/e 103, 105, 132, 134, 203 and 205 (Fig.9).

Larger more complicated peptides containing "troublesome" amino acids such as arginine, cystine, methionine and histidine, give f.d. spectra that are quite in accord with the generalization that direct simple bond clevage occurs in the backbone of peptide yielding complete sequence information.

The f.d.mass spectra of tetrapeptides are obtained with the molecular ion group $(M)^+$ being the most intense ions in the spectra. Sequence determination by low resolution f.d.m.s of the tetrapeptide H.Trp.Met.Asp.Phe.NH_2 is no more difficult than that encountered in the tripeptides. The spectrum contains ions corresponding to the terminal amino acids at m/e 188 and 164, the dipeptide fragments at m/e 318 and 278 and the tripeptide fragments at m/e 434 and 437. The overlap of the tripeptide and dipeptide fragments taken with the identification of the amino-and carboxyl-terminal amino acids and the amino acid analysis data gives the sequence H.Trp. Met.Asp.Phe.NH_2 for the tetrapeptide (Table XII). The spectrum is accounted for by the assumption of simple cleavage of the protonated parent molecule with initial bond rupture processes taking place at peptide bonds, followed by or concurrent with elimination of small neutral stable molecules (Fig. 10). In a recent report the c.i mass spectrum of this tetrapeptide was investigated (74). No molecular ion peak was observed and the amino terminal amino acid could not be located; an amino terminal dipeptide fragment ion (Trp ,Met), however, could be identified.

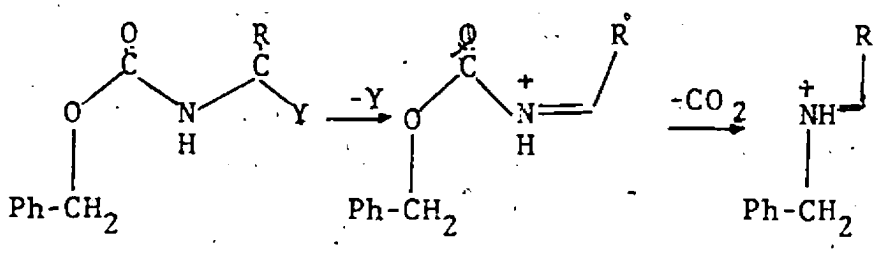
The outstanding features of the f.d. mass spectrum of the tetrapeptide H.Pro.Phe.Gly.Lys.OH.$COCF_3$ are the base peak at m/e 447 (peptide.$COCF_3$-$COCF_3$+H), the peak at m/e 543 (peptide.$COCF_3$) and a $(2M)^+$ ion at m/e 894. In f.d.m.s of many compounds a $(2M+1)^+$ ion complex is formed (111,128,136). However, in structures where hydrogen bonding between molecules is likely, it is possible to see $(2M)^+$ ion. It

has been suggested, according to energy requirements, that the $[2M]^+$ ion has the structure $[(M+1)^+ (M-H)]$ (136). The fragmentation pattern of the $[2M]^+$ ion is analogous to that of the $[M+1]^+$ ion. In addition fragments are lost which are unique to the $[2M]^+$ ion.

At lower heating currents (16 mA and 18 mA) the f.d. mass spectra of H.Pro.Phe.Gly.Lys,OH.$COCF_3$ exhibit very intense $[M+1]^+$ ion which is the base peak and very small fragment ions (Figures 11a and 11b). The fragments are all due to peptide bond cleavages or a loss of COOH or H_2O from the molecular ion; but the derivation of the sequence is not easy because of the lack of complete sequence information. A similar observation has been reported by Winkler and Beckey (62). At higher emitter heating currents (20-22mA), however, fragmentation is induced. The perturbation brought about by the necessity of higher emitter current to induce fragmentation of the sample produces a f.d. mass spectrum similar to some extent to e.i. mass spectra.

The determination of the sequence of Cbz-pentapeptide by f.d.m.s. would be more difficult. The peaks at m/e 591, 620, 663 and 681 which apparently arise from the peptide plus molecules of ethanol, water and/or ethylacetate would be confusing in the interpretation of the spectra. Coupled with an independent amino acid analysis and a correct deduction that the four highest peaks were not involved in the sequence of the peptide, the sequence of this pentapeptide could be determined. The ions of m/e 574 and 530 indicate a Cbz-penta-

peptide. The ions of m/e 107, 467, and 439 place the benzyloxycarbonyl group at the amino terminal position. Proline is placed at the carboxyl terminal position by the peak at m/e 114. The sequence peaks at m/e 171, 285, 382 and 439 indicate the sequence Gly.Pro.Leu.Gly.Pro.OH for the peptide. The peak at m/e 261 confirms the sequence Cbz.Gly.Pro. Rearrangement peaks often occur in the e.i. mass spectra of benzyloxycarbonyl derivatives of peptides (39). These rearranged ions arise as depicted in Scheme 3.



Scheme 3.

On the time scale for the f.d. source (which is several orders of magnitude shorter than that for the same instrument in e.i. mode) such a low frequency factor process (Scheme 3) could not occur.

The origin of the hydrogens which add to the peptides to produce $[M+1]^+$ and $[M+2]^+$ ions does not seem to arise from solvent. The pentapeptide applied to the emitter with ethanol - d_6 + water - d_2 as the solvent still produced $[M+1]^+$ and $[M+2]^+$ ions rather than $[M+2]^+$ and $[M+4]^+$ ions (Table XIV) which would have been the case if deuteriums were

transferred from the solvent molecules. Hydrogens transferred to the pentapeptide probably arise from either ethylacetate or more likely from another peptide molecule. The ionized pentapeptide appears to have in some cases, ethanol, water and/or ethylacetate bound to it. The ethanol appears to arise from ethylacetate rather than from solvent (Table XIV). The ethanol could arise from hydrolysis of ethylacetate on the emitter.

The f.d.m.s. of the disulfide bridge hexapeptide demonstrates that for this type of peptide very little molecular weight information can be obtained. A cleavage between the two sulfur atoms is detected with 100% abundance Sequence information in the $\left[\frac{M}{2}+1\right]^+$ fragment is complete (Table XV and Fig. 8.)

For the nonapeptide bradykinin a series of overlapping peptide fragments are produced (Fig. 15). Determination of the peptide sequence from these data would be difficult as the spectrum is much more complex. For larger peptides it will probably be necessary to label the amino terminal end of the peptide (115) before a unique sequence can be determined. In a recent report the c.i. mass spectrum of bradykinin was investigated (75). The bradykinin was vaporized by a rapid heating technique. A large number of fragments were obtained including many internal portions of the peptide which contained neither the carboxyl-terminal nor the amino-terminal amino acids. In f.d.m.s. only fragments containing either the carboxyl-terminal or amino-terminal

were obtained for all the peptides studied. The surprisingly high volatility of bradykinin may be due to the fact that three of the nine amino acids present contain secondary amino groups and therefore have no hydrogen attached to the amide group in the corresponding peptide linkage; fewer groups are available that can form hydrogen bonds. Previous attempts to determine the complete amino acid sequence of arginine derivatized, permethylated bradykinin by e.i. mass spectrometry have been unsuccessful. Lenard et al (45) determined the sequence of the four residues from the amino terminal and three residues at the carboxyl terminal but could not locate $Phe.^{5}$ Ser^{6}. Leclereq, Smith and Desiderio (64) sequenced residue 1 - 7 of bradykinin but did not observe carboxyl terminal sequence ions. A carboxyl terminal dipeptide fragment ion (Phe.Arg) was identified which, however, could not be sequenced.

The low resolution f.d.m.s. analysis of dipeptide mixture as a means of identifying the dipeptides may be inadequate. For the investigation of peptide mixtures using f.d.m.s. it is necessary to run several mass spectra at different emitter heating currents since peptides samples differ widely in their desorption behavior. With increasing emitter currents the f.d. mass spectra exhibit many peaks which may be due to complicated rearrangements and surface reactions (137) so that investigation becomes very difficult. Among the 441 dipeptides derivable in principle from 21 amino acids, there are a considerable number (Ala,Val), (Gly,Lue), (Ile,Gly), (Gly,Hyp) which have the same nominal molecular

weight so that investigation of the molecular ions alone may not be adequate. For the simple binary mixture of tripeptides the low resolution f.d.m.s. investigation produced nine possible sequences. The high number of possible sequences may be the result of a change over from one peptide in the sample to another during the interpretation. Furthermore, alternative sequences may arise by low resolution f.d.m.s. due to unit mass resolution.

The addition and loss of hydrogens by the peptides in surface reactions makes an independent amino acid analysis necessary if f.d.m.s. is to be used in peptide sequencing. Several amino acids (for instance, lysine at 146 and glutamic acid at 147, valine at 117 and proline at 115, threonine at 119 and valine at 117, aspartic acid at 133 and leucine or isoleucine at 131, methionine at 149 and glutamic acid at 147) have masses within one to two mass units. If valine and proline (or lysine and glutamic acid, threonine and valine, aspartic acid and luecine or isoleucine, methionine and glutatic acid) were in the same peptide investigation by low resolution f.d.m.s. may not be able to assign these amino acids a unique position in the sequence.

Solvent molecules being associated with peptide ion as is the case in the pentapeptide may cause problems in interpreting the spectra.

The volatility enhancement of peptides by f.d.m.s. is sensitive to inorganic impurities, chiefly sodium and potassium, which must be removed prior to dispersal of peptide

samples on the emitter wire. An attempt to remove completely sodium and potassium ions from peptide samples employing ion retardation chromatography, (Figs. 17 and 18) and ion exchange chromatography (Fig.19) was not successful. However, the application of gel chromatography using Bio Gel P-2 improved the mass spectra of large peptides (mol. wt. < 1000); smooth field desorption processes at relatively lower heating current were observed. Wood et al (138) have used macrocyclic ligands to improve the desorption characteristics of carboxylate salts. The interference of sodium salts in the rapid heating c.i.m.s. study of peptides has been reported by Friedman et al (75), suggesting that the phenomenon may not be inherent in the f.d. process but rather may be more general. The preclusion of f.d. m.s of organic compounds by sodium and/or potassium has been suggested to be the result of preferred adsorption and low volatility of an ionic layer between the anode and the organic sample (138).

Comparison of f.d. spectra of 'sodium free' peptide sample with f.d.m.s. of peptide - sodium chloride mixtures showed that sodium salt had no dramatic effect on the molecular ion (Table XXIII). These results conflict with our own results on the spectra of some sodium and potassium contaminated peptide samples as well as results reported by other workers (139, 140). The preclusion of f.d.m.s. of some peptide samples but not others by sodium may be due to the physical and chemical properties of the peptide, that is, the combination of amino acids in the peptide.

CHAPTER V

SUMMARY AND CONCLUSIONS

The use of f.d.m.s for sequencing of small peptides would appear to be valid in principle. The high temperature activiated emitter enables a choice to be made between either obtaining only molecular peaks or molecular peaks and producing fragments via thermally/field - induced degradation in the adsorbed layer together with field desorption.

Significant cleavages of the peptide backbone as well as volatility enhancement is apparent in the peptides studied. Larger peptides such as bradykinin can be sequenced with more effort.

Problems which arise are:

1) secondary processes which drain ion current from sequence - determining ions at the high emitter currents; secondary processes include loss of small neutral molecules such as water
2) addition or removal of hydrogen in surface reactions and solvent effects
3) interference from inorganic impurities chiefly sodium and potassium.

If these problems can be overcome f.d.m.s could provide a highly sensitive and rapid instrumental approach to small peptide sequence determination at the microgram level.

APPENDIX

Integral Mass Numbers Corresponding to Common Amino Acids

AMINO ACID (mol.wt.)	N-TERMINAL RESIDUE (A)	C-TERMINAL RESIDUE (B)	ASSOCIATED IONS (Loss of CO_2 from (A)
Glycine (75)	58	74	30
Alanine (89)	72	88	44
Valine (117)	100	116	72
Leucine + (131)	114	130	86
Serine (105)	88	104	60
Threonine (119)	102	118	74
Aspartic Acid (133)	116	132	88
Glutamic Acid (147)	130	146	102
Phenylalanine (165)	148	164	120
Tyrosine (181)	164	180	136
Tryptophan (204)	187	203	159
Lysine (146)	129	145	101
Histidine (155)	138	154	110

AMINO ACID (mol.wt.)	N-TERMINAL RESIDUE (A)	C-TERMINAL RESIDUE (B)	ASSOCIATED IONS (Loss of CO_2 from (A)
Proline (115)	98	114	70
Cysteine (121)	104	120	76
Methionine (149)	132	148	104
Arginine (174)	157	173	129

+ Not differentiated from Isoleucine

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